ANNUAL PROGRAM REVIEW

FOREST BIOLOGY

March 25-26, 1997

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Institute of Paper Science and Technology
500 10th Street, N.W.
Atlanta, Georgia 30318
(404) 894-5700
(404) 894-4778 FAX

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

FOREST BIOLOGY
PROJECT ADVISORY COMMITTEE

IPST Liaison: Jerry Pullman (404) 894-5307, FAX (404)894-4778
E-MAIL jerry.pullman@ipst.edu
PAC Chairman: Bob Lazar (609) 844-7236, FAX (609)844-7366
RAC Liaison: Richard Barker (609)896-1200, FAX (609) 844-7366

Mr. Wayne Boyd *(1999)
Technical Services Superintendent
Bowater Newsprint
Calhoun Woodlands Operations
5020 Highway 11
Calhoun, TN 37309
423-336-7458
423-336-7206 (FAX)

Dr. Mike Becwar *(1999) ***
Tissue Culture Center Leader
Westvaco Corporation
P.O. Box 1950
Summerville, SC 29484-1950
(803) 851-4769
(803) 875-7185 (FAX)
mbecwar@awod.com E-MAIL

Mr. Bob Kodrzycki **(Alternate)**
Tissue Culture Center Leader
Westvaco Corporation
P.O. Box 1950
Summerville, SC 29484-1950
(803)
(803) 875-7185 (FAX)
bkidrzyc@awod.com E-MAIL

Mr. Gary L. Cargile *(1998)
Wood Procurement Director
The Weston Paper and Manufacturing Co.
P.O. Box 539
2401 Prairieton Avenue
Terre Haute, IN 47802
(812)234-6688
SAME AS ABOVE (FAX)

Mr. Steve Coleman *(1995) ***
R&D Manager
Boise Cascade Corporation
P.O. Box 1060
DeRidder, LA 70634
(318)462-4030
(318)462-4085 (FAX)

Mr. Dan Morrow **(Alternate)**
Seed Orchard Manager
Boise Cascade Corporation
P.O. Box 37
Singer, LA 70660
(318)825-6329
318)825-6850 (FAX)

Mr. McDavid Hughes *(1996)
Manager Technical Control
Riverwood International USA, Inc.
Forest Research
P.O. Box 35800
West Monroe, LA 71294-5800
(318)362-2441 (FAX)

* The dates in ( ) indicate the final year of the appointment.

Revised 2/12/97

*** Company has an Alternate

Forest Biology Project Advisory Committee (Con't)

Dr. Robert Lazar *(1997) **(Chairman)** ***
Research Manager
Research & Development Division
Union Camp Corporation
P.O. Box 3301
Princeton, NJ 08543-3301
(609)844-7236
(609)844-7366 (FAX)

Dr. Steve Wann **(Alternate)**
Research Associate
Union Camp Corporation
P.O. Box 3301
Princeton, NJ 08543-3301
(609)844-7432
(609)844-7323 (FAX)

Mr. Mike Cunningham **(Alternate)**
Regeneration Group Leader
Union Camp Belville Greenhouse
Bellville, GA 30414
(912)739-4613

Mr. Gregory N. Leach *(1998) ***
Research & Development Manager
Champion International Corporation
P.O. Box 875
Cantonment, FL 32533-0875
(904)968-3050
(904)968-3027 (FAX)

Mr. James Hodges **(Alternate)**
Research & Development Manager
Champion International Corporation
P.O. Box 100
Silverstreet, SC 29145
(803)995-4842
(803)995-3915 (FAX)

Mr. John McCoy *(1998)
Forestry Business Unit Manager
Blandin Paper Company
P.O. Box 407
Grand Rapids, Minn. 55744
(218) 327-6386
(218) 327-6387 (FAX)

Dr. Lynne McCullough *(1998) ***
Manager Strategic Biological Sciences
Weyerhaeuser Company
WTC 1C2
Tacoma, WA 98477
(206)924-6031
(206)924-6736 (FAX)

Dr. Pramod Gupta **(Alternate)**
Weyerhaeuser Company
Mail Stop WTCG 30
Tacoma, WA 98477-0001
(206)924-6623
(206)924-6736 (FAX)

Mr. John Pait *(1998)
Manager Technical Services & Forest Resources
Georgia Pacific Corporation
133 Peachtree Street, N.E.
P.O. Box 105605
Atlanta, GA 30348-5605
(404)652-6688
(404)584-1467 (FAX)

Mr. James Rydelius *(1999)
Manager, Research & Silviculture
Simpson Paper Company
Simpson Timber Company
Redwood Operations
P.O. Box 1169
Arcata, CA 95521-1169
(707)822-0371
(707)822-4429 (FAX)

Revised 2/12/97

Forest Biology Project Advisory Committee (Con't)

Ms. Rene N. Settergren *(1999)
Group Planning & Resource Manager
Potlatch Corporation
105 Arch Street
Post Office Box 504
Cloquet, MN 55720
(218) 879-0439
(218) 879-3212 (FAX)

Dr. Brian J. Stanton *(1998)
Geneticist
James River Corporation
Northwest Fiber Supply
349 NW 7th Avenue
Camas, WA 98607-2042
(206)834-8342
(206)834-8252 (FAX)

Dr. Ronald A. Woessner *(1997)
Land & Forest Productivity Manager
The Mead Corporation
Columbus, GA 31908-9908
(706)576-6128
(706)571-7471 (FAX)

RAC Representative
Dr. Richard G. Barker
Corporate Director, R&D
Union Camp Corporation
P.O. Box 3301
Princeton, NJ 08543-3301
(609) 896-1200
(609) 844-7366 (Fax)

* The dates in ( ) indicate the final year of the appointment.

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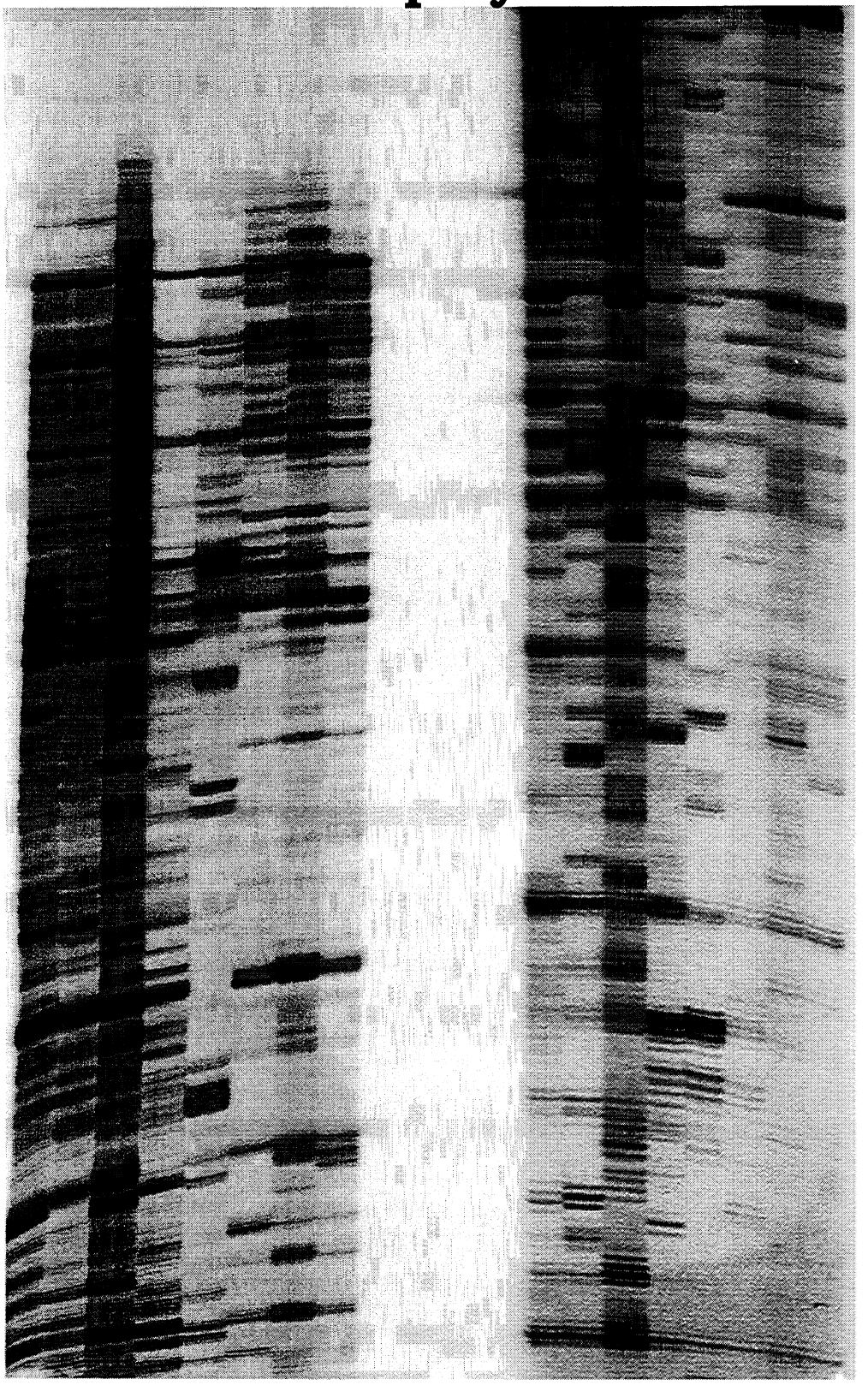
Differential Display Gel 4431-17



Mass Clonal Propagation of Improved Conifers

Status Report for
Project F010

Gerald Pullman
John Cairney
Gary Peter
Nanfei Xu
Barbara Johns
Shannon Johnson
Yolanda Powell
Paul Montello
Xiarong Feng
Kavita Namjoshi
Cielo Castillo
Alan Harris
Joanne Sumner

March 25-26, 1997

Institute of Paper Science and Technology
Atlanta, Georgia

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS
TECHNICAL PROGRAM REVIEW
February, 1996 - February, 1997

Project Title:	MASS CLONAL PROPAGATION OF IMPROVED CONIFERS
Project Code:	SFTWD
Project Number:	F-010
PAC:	Forest Biology
Division:	Chemical and Biological Sciences
Project Staff:	**Faculty**: Gerald Pullman, John Cairney, Gary Peter, open; **Postdocs**: Nanfei Xu;
Staff:	Barbara Johns, Shannon Johnson, Yolanda Powell, Paul Montello, Xiarong Feng, K. Namjoshi, C. Castillo, A. Harris, J. Sumner
FY 96-97 Budget:	$503,032
Supporting Research	
M. S. Students:	6
Ph.D. Students:	2
Govt. Grants:	$274,000

LONG RANGE RESEARCH AREA:

Virgin Fiber Supply

PROGRAM OBJECTIVE:

Develop methods suitable for commercial production of high quality somatic loblolly pine seedlings.

SUMMARY OF RESULTS:

Forest Biology Faculty brought in approximately $ 274,000 in outside funding during the past year for research related to F-010.

Confirmation of improved embryogenesis initiation protocol for immature seeds of high value loblolly pine. System working on cultures from many mother trees.

The Institute's first crop of loblolly pine somatic seedlings has been planted in the field. Germinated loblolly pine somatic embryos underwent successful conversion to growth in an open greenhouse and subsequent planting in a member company forest site. We currently have 35 plants in a field test.

Cryogenic storage system and methods for storage of loblolly somatic embryo cultures in liquid nitrogen are working well and becoming routine. Improved protocol has been developed which saves time, labor, materials and shows improved survival rates.

Metals analysis of full-term zygotic embryos and female gametophyte tissue from five seed sources is complete. Information begins to provide us with targets for our somatic embryos.

Differential display technique is working well in the laboratory. Results are beginning to show repeatable and comparable banding patterns for developmentally staged zygotic and somatic embryos of loblolly pine. Differences and similarities in gene expression between zygotic and somatic embryos are being observed for the first time.

Differential display has been applied successfully to single late stage loblolly pine embryos.

Fragments of the identified genes (cDNAs) from differential display have been isolated from two somatic genotypes and one zygotic genotype. These are being cloned and sequenced to determine the nature of the genes being induced at different developmental stages.

Collection made for staged zygotic embryos of loblolly pine ½ sub families, BC-1, and UC5-1036. This unique resource provides materials for analyses of staged embryos and female gametophytes.

Image analysis equipment purchased and operating. Will allow improved rapid evaluation and quantification of somatic embryos.

New approaches for improved somatic embryogenesis protocol and embryo quality integrated into program.
cDNA libraries from small amounts of tissue (early-staged embryos) made for studying gene expression
Antibody marker project begun to complement morphological based embryo evaluation and staging.

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS - INITIATION & CULTURE SURVIVAL

Gerald Pullman
Shannon Johnson
Yolanda Powell
Paul Montello
Alan Harris
Cielo Castillo
Kavita Namjoshi
Joanne Sumner

Summary

Results from Summer 1996 initiations confirmed last years initiation findings. Initiation rates have been improved by an order of magnitude compared to earlier years. Through a combination of 1/2 P6 Salts, activated carbon at 37.5-50 mg/l, extra added copper and zinc, 1.5% maltose, 2% myo-inositol, 500 mg/l case amino acids, 450 mg/l glutamine, Gelrite gelling agent at 1.5-2 g/l, and hormones consisting of 2 ppm NAA, 0.45 ppm BAP and 0.43 ppm Kinetin, initiation rates ranged from 0-47% across many genotypes (compared with last years range of 3-33%). This initiation improvement resulted from numerous experiments over the past 2.5 years which have been discussed in PAC reports for 1995 and 1996. During the Summer 1996 initiation trials over 13440 explants were placed on medium resulting in 765 initiations for an overall 5.7% success rate. Medium 505 still outperforms most modifications and resulted in an overall success rate of 7.4%. It is clear from our trials that much unexplained variation occurs from trial to trial with the same explant cone material. Maintenance of initiated cultures remains research focus point with 70-80% loss of cultures within the first several months after initiation.

Introduction & Results

The initiation of an embryogenic culture or embryo suspensor mass (ESM) is the first step in cloning the embryo(s) from a valuable conifer seed. The process in loblolly pine starts with an immature seed. The seed is sterilized and the seed coat removed to expose the ovule or female gametophyte which contains the early-staged embryo(s). The whole female gametophyte (megagametophyte) is placed on a chemically defined medium and incubated in the dark at 22-24 °C. The process of initiation then occurs in several phases: extrusion of zygotic embryos, formation of somatic embryos, and multiplication of embryogenic tissue into a culture. The results that we report are for successful progression through these three steps resulting in at least three visible somatic embryos emanating from a zygotic embryo(s).

Table 1 shows a summary of initiations for all media and all 1/2 sib families used during Summer 1996 initiation experiments. Cones were collected from eighteen families. All families contained early zygotic embryos at stages 2-4 that were suitable for experimentation. Sixteen families were

able to initiate cultures with initiation rates ranging from 3.3 to 19%. Table 1 compares initiation rates during 1995 and 1996 for twelve loblolly pine mother trees. Table 2 shows the composition of medium 505. Table 3 shows for each family tested, the percentage of initiation over all media, and culture survival after four to six months. Some families showed high levels of loss of cultures over time. Overall 5.7% initiation occurred across all families and 33% of the cultures initiated survived after four to six months. For comparison, similar data for 1995 is shown in Table 4. Significant improvement in culture maintenance is necessary. Culture survival of 50% is now one of our focus areas for ongoing research.

In order to better understand the mechanisms of culture loss, 68 new initiations were grown in medium 16 and their rates of survival and contamination tracked for a period of 15 weeks (Figure 1). This data begins to present a clear picture of how and when cultures are lost over time. Cultures begin the initiation and multiplication process in the initiation medium, but then about 70-80% do not continue growth after transfer to the maintenance medium. Some cultures lay dormant for several weeks on the maintenance medium before growth again begins. Cultures which show growth on the maintenance medium (16) then either continue to grow, become contaminated and are lost, or slowly lose vigor and eventually discontinue growth.

Table 1. Loblolly pine cone collection mother trees with 1995 and 1996 initiation rates on medium 505.

Tree Identification	Initiation - 1995		Initiation - 1996					
	All Media %	Media 505 %	Medium 505 % Individual Trials					Average
Boisie Cascade								
BC-1	1.0%		0.0%	13.3%				6.7%
BC-2	3.6%	10%	0.0%	0.0%	0.0%			0.0%
BC-3			3.0%	0.0%	6.7%			3.2%
BC-5	6.20%		10.0%	13.3%	6.7%	0.0%	16.7%	9.3%
BC-8	too early							
BC-9	6.4%	17%	27.0%	20.0%	6.7%	0.0%	0.0%	10.7%
Union Camp								
UC5-1036	16.4%	32%	17.0%	6.7%	0.0%			7.9%
UC7-1037	3.3%	10%						
UC7-1051	2.0%,		6.7%	6.7%	0.0%			4.5%
UC10-1027	10.0%	33%	27.5%	0.0%				13.8%
UC10-5	1.9%	3.3%						
UC10-33	6.7%	12%						
UC11-1055	1.7%		3.3%	3.3%				3.3%
UC11-1057			0.0%	43.3%	10.0%	16.7%	6.7%	15.3%
UC11-1066			20.0%	0.0%				10.0%
UC11-1069			10.0%	3.3%	0.0%			4.4%
Westvaco								
F2	12.1%	11%						
G2	stages too late							
H2	9.60%		16.7%	0.0%	0.0%	0.0%		6.7%
I2	3.8%	15%	0.0%	10.0%	0.0%			3.3%
J2	5.4%		7.0%					7.0%
K2			47.0%	10.0%	0.0%			19.0%
NC State								
NC7-1037self			0.0%					0.0%
Overall	6%	16%						7.4%

Table 2. Media composition for initiation medium 505 and maintenance medium 16.

Components	505	16
NH_4NO_3	200.0	603.8
KNO_3	909.9	909.9
KH_2PO_4	136.1	136.1
$Ca(NO_3)_2o4H_2O$	236.2	236.2
$MgSO_4o7H_2O$	246.5	246.5
$Mg(NO_3)_2o6H_2O$	256.5	256.5
$MgCl_2o6H_2O$	101.7	101.7
KI	4.15	4.15
H_3BO_3	15.5	15.5
$MnSO_4oH_2O$	10.5	10.5
$ZnSO_4o7H_2O$	14.69	14.4
$Na_2MoO_4o2H_2O$	0.125	0.125
$CuSO_4o5H_2O$	0.1725	0.125
$CoCl_2o6H_2O$	0.125	0.125
$FeSO_4o7H_2O$	13.9	6.95
Na_2EDTA	18.65	9.33
Maltose	15,000	0
Sucrose	0	30,000
myo-Inositol	20,000	1,000
Casamino acids	500	500
L-Glutamine	450	450
ThiamineoHCl	1.0	1.0
PyridoxineoHCl	0.5	0.5
Nicotinic acid	0.5	0.5
Glycine	2.0	2.0
2,4-D	0	1.1
NAA	2.0	0
BAP	0.45	0.45
Kinetin	0.43	0.43
Activated Charcoal	50	0
Gelrite	2,000	0
pH	5.7	5.7

Table 3. Overall initiation rates for all media tested and four to six month culture survival across sixteen 1/2 sib families during Summer, 1996.

Clone	# Initiations / Total	% Initiation	# Survived / Total Initiations	% Survival of Initiations
BC-1	18 / 480	3.8	4 / 18	22
BC-2	9 / 690	1.3	3 / 9	33
BC-3	56 / 720	7.8	17 / 56	30
BC-5	108/ 1320	8.1	53 / 108	49
BC-9	81 / 1320	6.1	44 / 81	54
UC5-1036	60 / 810	7.4	8 / 60	13
UC7-1051	31 / 750	4.1	5 / 31	16
UC10-1027	41 / 510	8.0	2 / 41	5
UC11-1055	31 / 810	3.8	4 / 31	13
UC11-1057	122 / 1350	9.0	76 / 122	62
UC11-1066	22 / 510	4.3	1 / 22	5
UC11-1069	21 / 960	2.2	0 / 21	0
WV-H2	59 /1080	5.5	23 / 59	39
WV-I2	16 / 840	1.9	0 / 16	0
WV-J2	27 / 240	11.3	0 / 27	0
WV-K2	63 / 810	7.8	9 / 63	14
NC7-1037 self	0 / 240	0	0	0
Overall Totals	**765 / 13440**	**5.7**	**249 / 765**	**33**

Table 4. Overall initiation rates for all media tested and six month culture survival across sixteen 1/2 sib families during Summer, 1995.

Clone	# Initiations / Total	% Initiation	# Survived / Total Initiations	% Survival of Initiations
BC-1	5 / 480	1.0	3 / 5	60
BC-2	15 / 420	3.6	0 / 15	0
BC-3	37 / 469	7.9	3 / 37	8.1
BC-5	15 / 240	6.2	1 / 15	6.7
BC-8	Embryos too early or shriveled.		-	-
BC-9	27 / 420	6.4	11 / 27	41
UC5-1036	87 / 529	16.4	25 / 87	29
UC7-1051	5 / 240	2.0	3 / 5	60
UC7-1037	7 / 210	3.3	0 / 7	0
UC10-5	8 / 420	1.9	0 / 8	0
UC10-33	28 / 420	6.7	8 / 28	29
UC10-1027	45 / 450	10.0	5 / 45	11
UC11-1055	4 / 240	1.7	0 / 4	0
WV-F2	87 / 714	12.1	21 / 87	24
WV-G2	Stages too late.	-	-	-
WV-H2	23 / 240	9.6	11 / 23	48
WV-I2	17 / 450	3.8	4 / 17	24
WV-J2	26 / 480	5.4	2 / 26	7.7
Overall Totals	**436 / 6422**	**6.8**	**97 / 436**	**22**

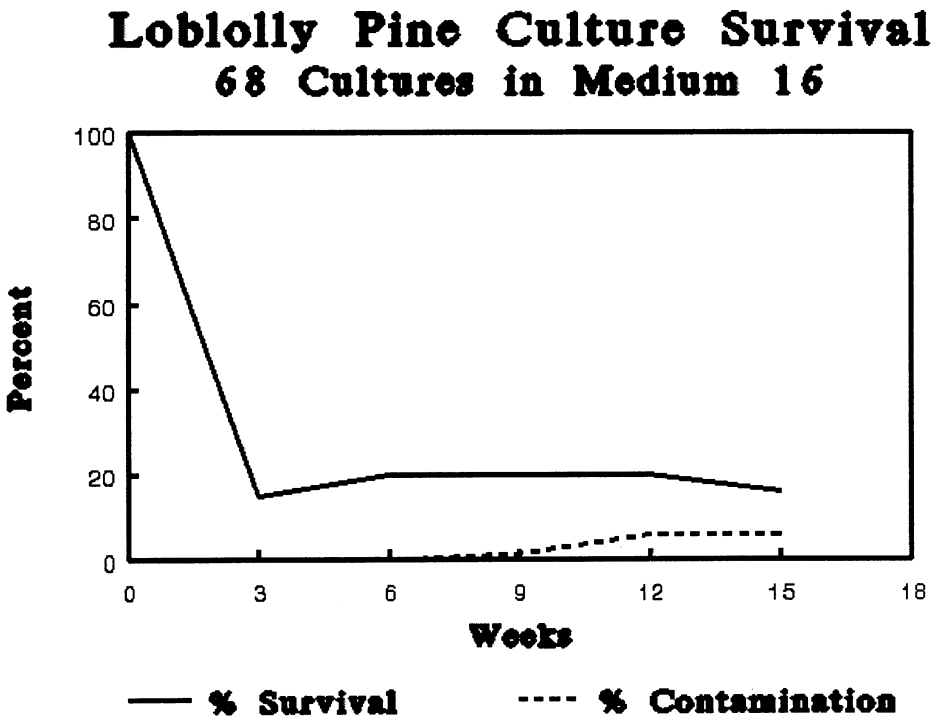


Figure 1. Survival and contamination % over 15 weeks for loblolly pine embryogenic tissue grown in medium 16.

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS: LONG-TERM STORAGE OF LOBLOLLY PINE SOMATIC EMBRYO CULTURES IN LIQUID NITROGEN

Jerry Pullman, Paul Montello, Yolanda Powell, and Cielo Castillo

Summary: Ninety one genotypes of loblolly pine have been placed in cryogenic storage using the method of Kartha et al. (1988). To date 81 genotypes have completed testing for survival after at least 3 days in liquid nitrogen. Of these genotypes 74% showed recovery. A modified protocol has been developed which currently shows improved survival over the standard protocol. In addition, this modified protocol shows faster embryo recovery, lower labor costs, and lower supply costs.

Introduction

There are many challenges that face the commercialization of micropropagated elite and superior lines of forest trees. As is true with traditional tree breeding, the long life cycle of trees presents a great challenge to genetic improvement. Any putatively superior material that is cultured, cloned and possibly even genetically engineered must undergo field tests which may require several decades. In the duration, the embryogenic cultures must be stored in the laboratory. In addition, a commercial laboratory propagating high value trees by embryogenesis will require flexibility to start and stop production of particular genotypes when desired.

The long term maintenance of forest tree tissue cultures faces many problems. First, it consumes a great deal of labor and supplies. Second, routine transfers to fresh medium give multiple opportunities for contamination of the culture. Somaclonal variation poses a third problem associated with long term maintenance. This could also include the loss in expression of transgenes in genetically engineered cultures. Fourth, many cultures exhibit a loss of embryogenic potential over time. Lastly, in some cases, prolonged culture on maintenance medium can lead to mortality and thus loss of superior germplasm.

In the case of IPST's loblolly pine embryogenic system, maintenance requirements of over a hundred genotypes of embryogenic pine cultures consumes tremendous amounts of labor and supplies. If these cultures could be stored in liquid nitrogen several benefits could accrue. The most important benefit would be the freeing up of laboratory staff time from maintenance chores which could be redirected towards new research. A known effect of storage at ultra-low temperatures, such as that of liquid nitrogen (-196°C), is the halting of all metabolic activity. Consequently, cryopreservation could minimize somaclonal variation, loss of embryogenic potential, and deterioration of natural and genetically engineered lines. Lastly, frozen cultures could be held indefinitely, whereas their non-frozen counterparts have historically sustained

almost 80% mortality after less than a year. In this report we will describe our efforts to cryopreserve over 91 genotypes of loblolly pine early stage embryo cultures and experiments designed to optimize this process.

Materials and Methods

Water is the major component of all living cells and must be present in order for chemical reactions to occur within a cell. During cryopreservation, the water changes to ice and cellular metabolism ceases. The formation of microscopic ice crystals can fracture cellular membranes leading to cell death. Dehydration also occurs changing the concentration of salts and other metabolites, causing an osmotic imbalance which can be detrimental to cell recovery. These detrimental effects can be minimized by controlling the rate of cooling, using cryo-protective agents, and maintaining appropriate storage temperatures and rates of re-warming.

To limit the amount of cellular water available to form microscopic ice crystals we employed an osmotic pretreatment. 30 ml of loblolly pine tissue culture grown on Medium 16 (Table 1) was placed in 120 ml liquid maintenance medium supplemented with 0.2M sorbitol (Medium 555, Table 1) for 24 hours. Then the cells were transferred to 120 ml of liquid maintenance medium supplemented with 0.4M sorbitol (Medium 556, Table 1) for an additional 24 hours. Prior to cryopreservation cells were exposed to dimethyl sulfoxide, a compound known to minimize rupture of cellular membranes. The cell suspensions were allowed to settle after which 53 ml of liquid medium was pipetted out leaving a final volume of 97 ml. The cells were resuspended in a 1000 ml flask and 1.2 ml of filter sterilized DMSO was added. The flask was placed in an ice bucket for 15 minutes before dispensing an additional 1.2 ml DMSO. Fifteen minutes later a final aliquot of 1.1 ml DMSO was pipetted into the flask to bring the final cryoprotectant concentration to 5%. Then approximately 1.8 ml of the suspension was pipetted into 35 2 ml Nalgene cryogenic vials and placed into a programmable freezer.

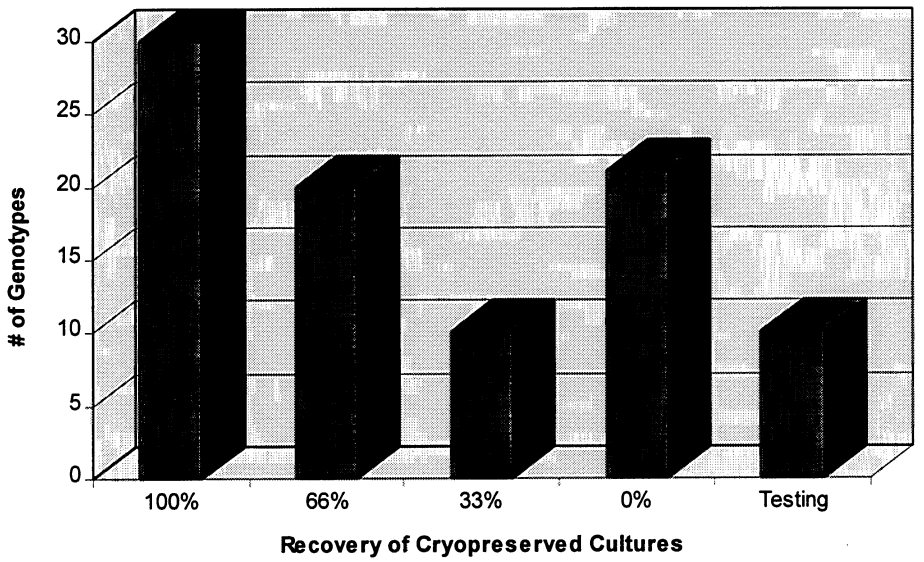
The pine cell cultures underwent freezing to -35°C at a rate of approximately 0.33°C min^{-1}. Then the cryogenic vials were transferred to a cryobox and submerged in the liquid nitrogen storage chamber.

To thaw cells the vials were removed from the liquid nitrogen storage chamber and thawed in a 37°C water bath for approximately 5 minutes. The exterior of the cryovials were sprayed with 70% ethanol and dried with a Kimwipe. The cryovials were opened, flamed, and the contents poured onto a sterile black filter disc (4.25cm) on a petri dish (100x15mm) containing 20 ml semi-solid Medium 16. After one hour the filter disc was moved onto a petri dish of fresh medium. Eighteen hours later, the filter disc was again transferred to a petri dish of fresh loblolly pine maintenance medium. The plates were stored in the dark and periodically observed for culture growth.

Results

This method was employed to cryogenically store 91 loblolly pine genotypes. Of the 81 lines that have been evaluated 71 have displayed growth defined as expansion of clear, translucent finger-like tissue (Figure 1). Three cryovials were removed from storage for each genotype. Regrowth was observed on all three plates for 30 genotypes. Twenty embryogenic lines displayed recovery on two out of three plates. Ten lines only recovered on one out of three samples thawed. No regrowth was observed with 21 genotypes of loblolly pine. We are in the process of testing the recovery of the remaining 10 embryogenic lines. Overall, recovery as observed on at least one of three samples was 74%.

Figure 1.



Improved Technique

The above described protocol was modified to optimize the recovery of loblolly pine embryogenic cultures. Preliminary experiments revealed that it may be possible to eliminate the first 24 hour osmotic pretreatment which uses 0.2M sorbitol.

Experiment 1: To test this hypothesis we used two genotypes (260 & 261), two treatments (0.2M sorbitol pretreatment 24 hours followed by 0.4M sorbitol pretreatment 24 hours; or just the by 0.4M sorbitol pretreatment for 24 hours alone). Samples were thawed on two different days (separated by one day) and for both of these blocks ten replications were performed.

Experiment 2: The same two pretreatment regimes used in Experiment 1 were used on six genotypes(195, 230, 247, 255, 256, 261). Five replications were performed.

Experiment 3: The same pretreatment regime used in Experiment 1 was used on four different genotypes (3ALP, 6ALP, 22, 48). Ten replications were used.

Results

Experiment 1: When we tested this modification on two genotypes of loblolly pine it was found that a 0.4M sorbitol pretreatment 24 hours prior to cryopreservation enhanced recovery post thaw. Approximately four times as many survival colonies were noted with the modified protocol compared to the standard protocol (Figure 2). In addition, quicker recovery was accomplished by eliminating the first osmotic pretreatment period.

Experiment 2: Figure 3 shows that three lines performed slightly better with the modified protocol. One line recovered equally well with either protocol. Two lines displays slightly worse recovery with the modified protocol. When considering the standard error bars five lines recovered as well as if not better that the control while only one is significantly worse.

Experiment 3: The final time the experiment was repeated, all four genotypes recovered from storage in liquid nitrogen equally well using either protocol (Figure 4).

Statistical Analysis: When data from all three experiments was combined and analyzed by ANOVA using Statgraphic Software, Line was highly significant. Furthermore, Treatment was significant at the p=0.07 level. The interaction between Line and Treatment was also highly significant (Table 2).

The data suggests that although genotypic differences in ability to survive cryopreservation exist, using the new protocol will allow loblolly pine suspension cultures to be retrieved as well as if not better than the control protocol. Furthermore, the savings in time and supplies are realized by using the new protocol.

Reference

K. K. Kartha. L.C. Fowke, N. L. Leung, K. L. Cashwell and I. Hakman (1988) Induction of somatic embryo and plantlets from cryopreserved cell cultures of white spruce (*Picea glauca*). Journal of Plant Physiology 132: 529-539.

Table 1.

	Media mg/l		
Components	16	555	556
NH_4NO_3	603.8	603.8	603.8
KNO_3	909.9	909.9	909.9
$MgSO_4 \cdot 7H_2O$	246.5	246.5	246.5
KH_2PO_4	136.1	136.1	136.1
$Ca(NO_3)_2 \cdot 4H_2O$	236.2	236.2	236.2
$Mg(NO_3)_2 \cdot 6H_2O$	256.5	256.5	256.5
$MgCl_2 \cdot 6H_2O$	101.7	101.7	101.7
KI	4.15	4.15	4.15
H_3BO_3	15.5	15.5	15.5
$MnSO_4 \cdot H_2O$	10.5	10.5	10.5
$ZnSO_4 \cdot 7H_2O$	14.4	14.4	14.4
$Na_2MoO_4 \cdot 2H_2O$	0.125	0.125	0.125
$CuSO_4 \cdot 5H_2O$	0.125	0.125	0.125
$CoCl_2 \cdot 6H_2O$	0.125	0.125	0.125
$FeSO_4 \cdot 7H_2O$	6.95	6.95	6.95
Na_2EDTA	9.33	9.33	9.33
Sucrose	30,000	30,000	30,000
D-Sorbitol	--	36,440	72,880
myo-Inositol	1,000	1,000	1,000
Casamino acids	500	500	500
L-Glutamine	450	450	450
Thiamine•HCl	1.0	1.0	1.0
Pyridoxine•HCl	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5
Glycine	2.0	2.0	2.0
2,4-D	1.1	1.1	1.1
BAP	0.45	0.45	0.45
Kinetin	0.43	0.43	0.43
pH	5.7	5.7	5.7

Table 2. Analysis of Variance - Type III Sums of Squares

Source of Variation	Sum of Squares	d.f.	Mean square	F-ratio	Sig. level
MAIN EFFECTS					
A: Line	14368.898	10	1436.8898	7.807	0.0000
B: Treatment	630.048	1	630.0481	3.423	0.0659
INTERACTIONS					
AB	4908.0052	10	4908.0052	2.666	0.0046
RESIDUAL	34235.502	186	184.06184		
TOTAL (CORRECTED)	57159.519	207			

13 missing values have been excluded.
All F-ratios are based on the residual mean square error.

Figure 2. Treatment B (control) underwent 24 hours in 0.2M sorbitol followed by 24 hours in 0.4M sorbitol. Treatment C was subjected to only 24 hours in 0.4M sorbitol before freezing.

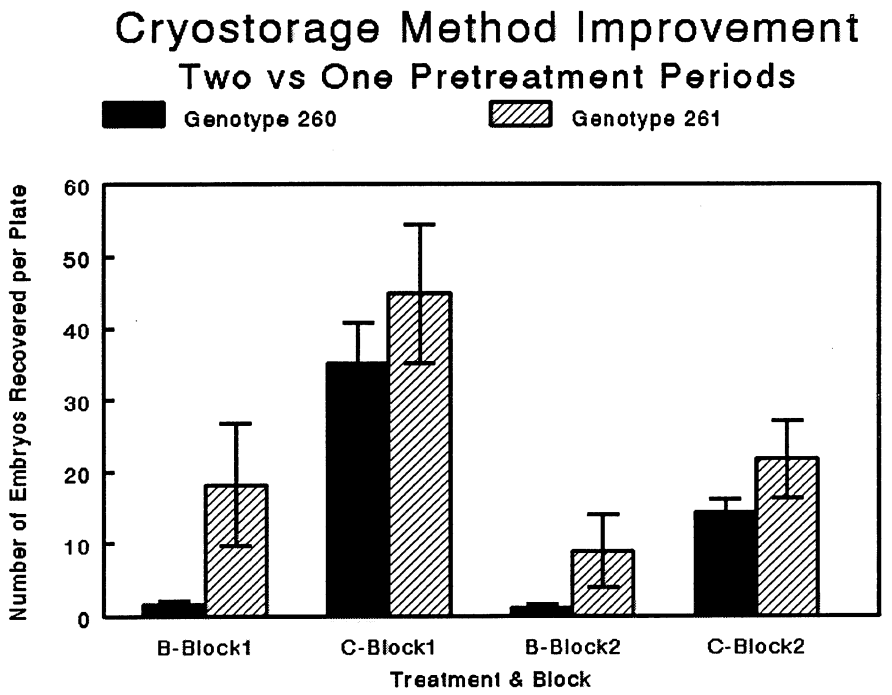


Figure 3.

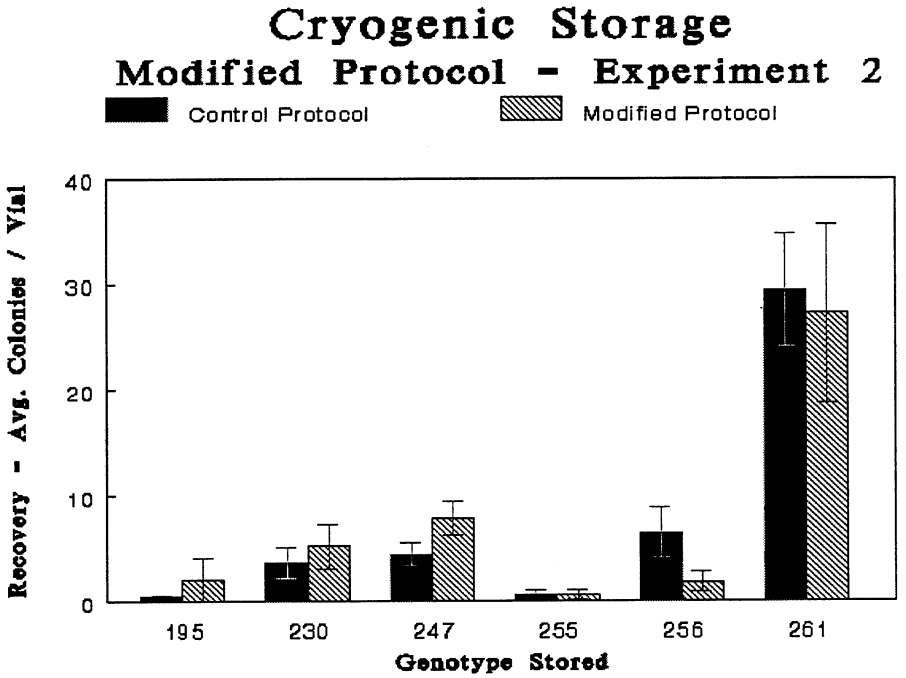
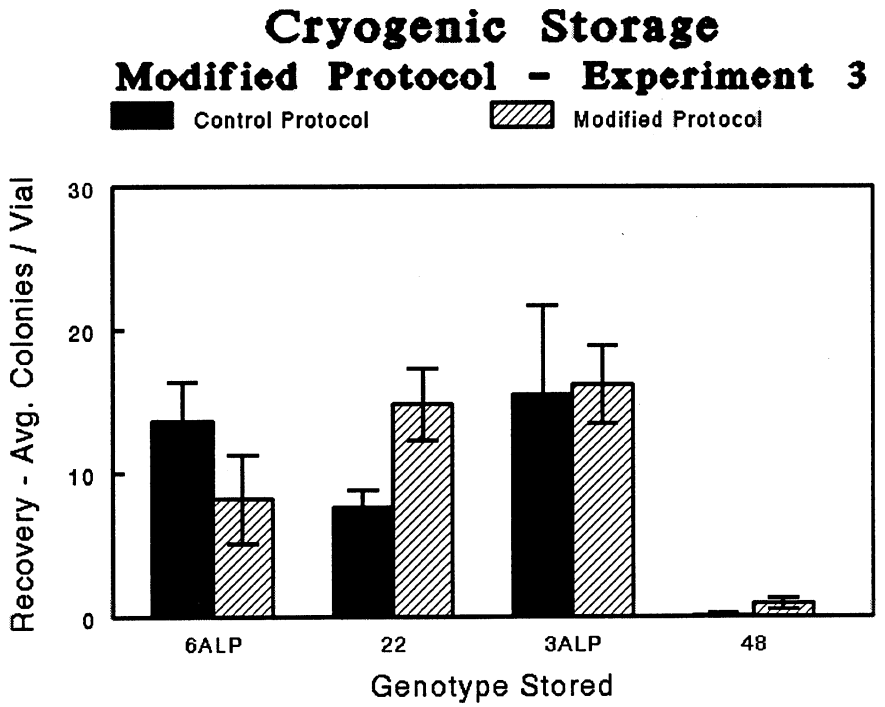


Figure 4.


Cryogenic Storage
Modified Protocol - Experiment 3
Control Protocol
Modified Protocol
Recovery - Avg. Colonies / Vial
30
20
10
0
6ALP
22
3ALP
48
Genotype Stored

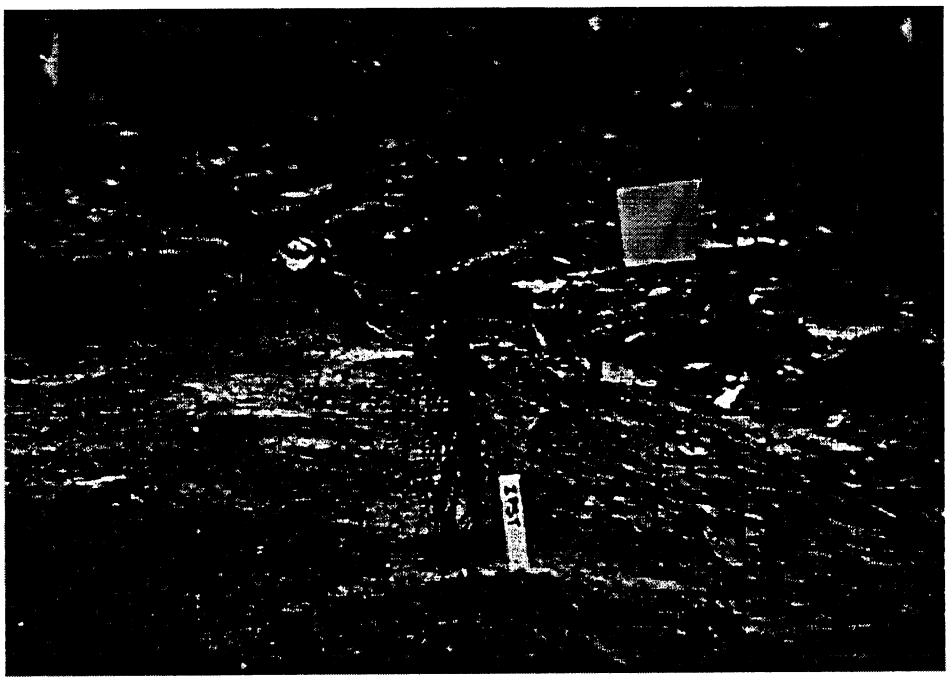
MASS CLONAL PROPAGATION OF IMPROVED CONIFERS: FIELD ESTABLISHMENT OF SOMATIC EMBRYO DERIVED LOBLOLLY PINE SEEDLINGS

Jerry Pullman, Paul Montello, Mike Cunningham

Summary: On January 22, 1997 thirty five somatic embryo derived loblolly pine seedlings initiated in summer 1994 from open pollinated ovules from tree UC10-1003 were established in a field plot at the Union Camp Ogeechee Forest in Tattnall County Georgia.

Loblolly pine seeds that originated from tree UC10-1003 of the Union Camp Corporation were initiated during 1994 initiation trials. The somatic embryos that resulted subsequently were allowed to undergo conversion and germination. The seedlings spent approximately 1 year in the greenhouse. This past winter 35 of these seedlings were delivered to the Union Camp Bellville Georgia location. On January 22, 1997 they were established in the field. The study was laid out in four rows. Rows 1-3 have 9 trees whereas Row 4 has 8 trees. The spacing between seedlings is 10'x6'. The study plot is marked with a post at each corner and a flag pin at each tree.

We would like to thank Mike Cunningham, Randy Purvis, and Jerome Martin at Union Camp for the establishment of these seedlings.



MASS CLONAL PROPAGATION OF IMPROVED CONIFERS: PROGRESS IN PROTOCOL DEVELOPMENT & TARGETS

Jerry Pullman
Shannon Johnson
Yolanda Powell
Paul Montello
Elizabeth Bittner
Clay Juckett

Summary

During the 1995 Summer approximately 440 cultures of loblolly pine were initiated. Since we reported in previous PAC Meetings that loblolly pine cultures often loose their ability to form cotyledonary embryos over time, these new cultures provided an opportunity to determine how well our protocols work.

Twenty-five cultures which contained enough embryogenic tissue for starting liquid cultures were chosen for testing in one of our liquid maintenance protocols (medium 16) and one of our development and maturation protocols (medium 240). Liquid cultures were started as reported previously. Settled cell volumes and embryo stage ratings were taken weekly (Table 1). After five or more subcultures, settled cells were plated with one ml per plate on development medium 240. Cotyledonary embryo yield results are shown in Table 1. Most genotypes which produced stage eight embryos have been tested for germination. Embryos are considered germinated if they show epicotyl and root growth.

Data in Table 1 show that out of 25 cultures tested, 18 (72%) were able to start liquid cultures. Two of the seven cultures that did not start were re-tested for the ability to start a liquid culture. Both of these cultures started liquid cultures bringing the overall liquid culture success rate to 80%. The remaining five cultures had stopped growing in the maintenance medium and were no longer available. An additional observation is that during the time period that the 25 cultures were being evaluated, some of the mother cultures maintained on solid medium 16 declined and stopped growth while the liquid cultures continued. It thus appears that growth in liquid 16 can somewhat prevent or delay the loss of a culture. Average weekly multiplication rates over five weeks ranged from 1.5 to 4.7 times increase per week. Average weekly embryo stage ratings varied from 1-2.9 (see embryo classification system reported in the March 21, 1994 PAC Report). Maximum stage ratings of at least 2 were seen in 5/25 cultures (25%). The maximum stage rating is important because previous observations showed that liquid cultures with stage ratings of two or higher had the best chance of producing cotyledonary embryos. Fourteen out of 25 genotypes (56%) produced cotyledonary embryos between stages 6-8. Genotypes with stage 7-8 embryos and with enough embryos for germination testing were tested for germination on medium 55. Four out of the seven genotypes tested showed 1-33% germination. All nineteen of the liquid grown cultures were placed in cryogenic storage. Eighteen of these cultures (95%) have shown

successful survival and retrieval from cryogenic storage. It is interesting to note that cultures initiated in late Summer 1995 and placed in cryogenic storage in early Summer 1996 show higher survival rates than older cultures. Out of the 25 genotypes used to start the experiment 18/25 are now retrievable from cryogenic storage.

Tables 2 and 3 show various media compositions and an overall summary of loblolly pine somatic embryogenesis at IPST. Major progress has been made in the areas of initiation, maintenance, embryo development, germination, conversion, and cryogenic storage. However, there are many challenges ahead particularly in the area of embryo quality. Embryo quality and vigor must be improved during all stages for the somatic embryogenesis process to become commercial. With the successful storage of cultures in liquid nitrogen shortly after initiation we hope to prevent any culture decline over time, decrease the labor to maintain cultures, and have available a bank of cultures with known histories of performance in the somatic embryogenesis process. Research could then focus on the particular step or embryo stage where improvement is needed.

Table 1. Loblolly pine culture performance: starting liquid cultures, weekly growth rate as settled cell volumes, embryo stage in liquid culture (medium 16), and maximum stage rating in development and maturation medium (medium 240).

Culture #	Origin	Weekly Settled Cell Volume (5 wk avg.)	Liquid Stage Rating - (5wk average/ maximum rating)	Cotyledonary embryos / ml plated cells, repeated platings	Germination (epicotyl & root growth) on med 55	Cryogenic Survival
245	UC5-1036	2.4	1.7 / 2	2.6, 15.8	Not Tested	3/3
246	UC5-1036	1.6	1.4 / 2	0,0		1/3
247	UC5-1036	1.8	1.7 / 2	1.2, 2.5, **5**	Not Tested	3/3
248	BC-3	No Start[2]				
249	WV-F2	3.2	2.0 / 2.5	9.6, 0	Not Tested	3/3
250	WV-F2	3.6	1.7 / 2	0, **0**		2/3
251	WV-F2	No Start[1]		0, **23**		3/3
252	BC-9	2.7	2.5 / 3	7.8, 50+, **25**	Not Tested	2/3
253	UC10-33	4.6	1.7 / 2	0.8	6 weeks	3/3
254	UC10-33	2	1.8 / 2	0, **11**	4.5 weeks	3/3
255	BC-9	1.8	2 / 2	18.6, 12.3, 15	0/70 (0%)	1/3
256	WV-F2	1.3	1.8 / 2	7.4	Not Tested	0/3
257	UC10-33	3.2	2.1 / 3	8.4, 10.5	1/70 (1%)	3/3
258	UC10-5	No Start[2]				
259	BC-9	2.1	2.9 / 3	39.8, 23.6, 41.6	0/500 (0%)	3/3
260	BC-3	1.5	2 / 2	8.0, 21.2	**0/20 (0%)**	2/3
261	UC5-1036	1.4	2.2 / 3	40.8, 33.8	16/349 (5%)	3/3
262	WV-I2	1.9	1 / 1	0	Not Tested	3/3
263	UC10-5	No Start[2]				
264	WV-F2	No Start[2]				
265	UC5-1036	No Start[1]				
266	UC10-33	2.3	2.7 / 3	10.8, 1.3	33/100 (33%)	3/3
267	UC10-33	2.4	1.6 / 2	24, 4.5	20/151 (13%)	3/3
268	UC5-1036	4.7	1.5 / 1.5	1.6, 3.7	0/20 (0%)	3/3
269	BC-2	No Start[2]				
Average per step		72% 1st Start80% 1st & 2nd Start	2.5 / 2.2	Yield per ml cells = 10.6 cotyledonary embryos	6.5% **4/8** germ	18/19 (95%)
Success / 25 Cultures		80%		14/25 (56%)	16%	72%

[1]/ Upon a second try liquid cultures were successfully started.

[2]/ Tissue was unavailable for a second attempt to start liquid cultures.

Table 2. Media compositions for initiation, maintenance, development, and germination media.

Components	505	16	240	55
		mg/liter		
NH4NO3	200	603.8	200	206.3
KNO3	909.9	909.9	909.9	1170
KH2PO4	136.1	136.1	136.1	85
Ca(NO3)2•4H2O	236.2	236.2	236.2	0
MgSO4•7H2O	246.5	246.5	246.5	185.5
Mg(NO3)2•6H2O	256.5	256.5	256.5	0
MgCl2•6H2O	101.7	101.7	101.7	0
KI	4.15	4.15	4.15	0.415
H3BO3	15.5	15.5	15.5	3.1
MnSO4•H2O	10.5	10.5	10.5	8.45
ZnSO4•7H2O	14.69	14.4	14.4	4.3
Na2MoO4•2H2O	0.125	0.125	0.125	0.125
CuSO4•5H2O	0.1725	0.125	0.125	0.0125
CoCl2•6H2O	0.125	0.125	0.125	0.0125
FeSO4•7H2O	13.9	6.95	6.95	13.93
Na2EDTA	18.65	9.33	13.9	18.65
Maltose	15,000	0	20000	0
Sucrose	0	30,000	0	20,000
PEG 8,000	0	0	130,000	0
myo-Inositol	20,000	1,000	20,000	100
Casamino acids	500	500	500	0
L-Glutamine	450	450	450	0
Thiamine•HCl	1	1	1	1
Pyridoxine•HCl	0.5	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5	0.5
Glycine	2	2	2	2
2,4-D	0	1.1	0	0
NAA	2	0	0	0
BAP	0.45	0.45	0	0
Kinetin	0.43	0.43	0	0
ABA			5.2	0
Activated Charcoal	50	0	0	2,500
Gelrite	2,000	0	2,500	
TC Agar	0	0	0	8,000
pH	5.7	5.7	5.7	5.7

Table 3. Summary of loblolly pine somatic embryogenesis at IPST with past, current, and target performance for major steps in the process.

TISSUE CULTURE STEP	1991	1992	1993	1994	1995	1996	Target
Initiation	<3%	<3%	<1%	<1%	16%	**7.4%**[4]	35%
Maintenance							
Survival on gelled media							
Short-term (<6 months)						20%	50%
Long-term (>6 months)			83%[1]				
Growth (G=gelled, L=liquid)	G-2x/mo	G-2x/mo	L2.5/wk	L2.5/wk	L2.5/wk	L2.5/wk	OK
Cryogenic Storage						**74%**	80% liq Cultures
Liquid Culture Embryo Quality			25%[2]			64%[3]	OK
Embryo Maturation							
Yield Stage 6+ /per ml cells)	<1	<1	<1	10+	10+	10.6	25+ High Quality
Stage (quality)	7	7	7	8	8	6-8	9.4
#Genotypes	~3	~3	~3	~5	~5	14/25 (56%)	50% liq cultures
Germination (shoot /root)	0%	0%	0%	30%	30%	0-33%	75% of embryos
Germination - Genotypes				1	1	4/25 (16%)	50% liq cultures
Acclimation -% of embryos					94%		80% of germinants
Acclimation - Genotypes					1	2	50% liq culture

[1]/ Already existing cultures, previous survival 6 months or longer. [2]/ Percent of cultures which produce early-stage embryos of stage 2 or better in liquid medium 16, starting cultures maintained in maintenance medium longer than 1 year. [3]/ Percent of cultures which produce early-stage embryos of stage 2 or better in liquid medium 16, starting cultures were recently initiated with growth in initiation or maintenance medium less than 3 months. [4]/ **Data in bold format represent changes within the past six months.**

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS - ELEMENTAL ANALYSIS OF ZYGOTIC FEMALE GAMETOPHYTE AND EMBRYO TISSUES

Gerald Pullman
Mike Buchanan
Yolanda Powell

Summary

Full term seeds from loblolly pine were dissected to remove the seed coat, integument, and nucellus and divided into the female gametophyte and embryo tissue. Tissue was analyzed in replicate for major and minor elements by use of Inductively Coupled Plasma Emission Spectroscopy (ICPES). Five sources of seed were analyzed in order to determine an approximate range of natural elemental composition. Variation between the five seed sources was minimal providing future elemental composition targets for somatic embryos.

Introduction

The nutritional, osmotic, and hormonal environments surrounding an embryo are well known to control embryo growth. Optimization of these environments is critical for the growth and development of high quality, vigorous somatic embryos. In optimizing the nutritional environment for somatic embryo development, we propose that analysis of the elemental contents of the female gametophyte and the zygotic embryo will provide models and targets for the development medium and somatic embryo elemental compositions respectively.

Following is a tentative step by step plan of how we plan to use analysis information of zygotic and female gametophyte tissue to improve our somatic embryo protocol. So far, we have completed the first step and are working on step 2.

Step 1. The first phase of this research is to determine how wide or narrow the natural or acceptable range of elemental composition is for female gametophyte and embryo tissue. We decided to initially use full term loblolly pine seed due to its availability and ease in obtaining the necessary sample weights for analysis. This data provides elemental targets for the somatic embryo and potentially for the medium.

Step 2. Analyze somatic embryos for elemental composition. Use somatic embryos as close to maturation as we can currently grow. Compare to target range for each element in analysis.

Step 3. Modify development and maturation medium based on review of the elemental composition data. Grow new somatic embryos on modified medium, observe somatic embryos for morphological improvements, analyze somatic embryos for new elemental composition and fit to target range.

Step 4. Analyze female gametophyte and zygotic embryos for each developmental stage to provide a time course of elemental composition during embryo and female gametophyte developments. (Note, some methods development is necessary to analyze the small amounts

available of very early stage zygotic embryos. Example, twenty stage one embryos have a dry weight of approximately 0.3 mg)

Step 5. Modify initiation, maintenance, and maturation medium based on the pattern of elemental change in developing female gametophyte and zygotic embryo tissues. Observe somatic embryos for morphological improvements, analyze somatic embryos grown in modified medium for new fit to elemental target range.

Five sets of cones or seed lots of ½ sib materials from four locations and seed orchards were analyzed for embryo and female gametophyte elemental composition. In order to make seed and embryo dissection easier, cones were requested at the end of seed development just prior to the seed drying process. With seed collection just prior to drying any potential elemental loss due to imbibition was eliminated and seeds were easier to open and remove embryos. Seed collections were obtained as follows.

BC-1	Boisie Cascade 1995 seed produced in a seed orchard near Lake Charles, Louisiana.
UC5-1036	Union Camp 1995 seed produced in a seed orchard near Bellville, GA.
UC10-1003	Union Camp 1995 seed produced in a seed orchard near Rincon, GA.
UC10-14	Union Camp 1995 seed produced in a seed orchard near Rincon, GA.
7-56	Westvaco 1995 seed produced in a seed orchard near Summerville, SC

Materials and Methods

Collected embryos were dried overnight at 70 °C and stored in a freezer. Pre-dried specimens of Loblolly Pine embryos and gametophytes were submitted for elemental analysis in individually labeled polyethylene micro-centrifuge tubes. To enable these samples to be analyzed for trace metals by Inductively Coupled Plasma Emission Spectroscopy (ICPES), the constituent metals present in the solid sample material were dissolved in an aqueous acid solution. The preparation procedure followed is outlined below:

1. An aliquot (approximately 50 mg) of pre-dried sample, was weighed into a new, labeled, graduated, screw-cap, polyethylene centrifuge tube. The weight of the sample was recorded to the nearest 0.1 mg.

2. Five milliliters (5.0 ml) of high purity concentrated Nitric Acid (EM Science TracePur Plus Instrumental Grade) were added to each tube, then capped, and allowed to stand at room temperature for six hours in a fume hood.

3. The tube was uncapped and two milliliters (2.0 ml) of high purity 30% Hydrogen Peroxide (J.T. Baker Ultrex Ultrapure Reagent Grade) were added. The tube was capped, inverted twice to mix the contents, and vented to release any evolved oxygen. The cap was loosely screwed on the tube to prevent pressure buildup from evolved oxygen. Each sample was allow to digest at room temperature for 24 hours in a fume hood.

4. At the end of the digestion period, ultrapure reagent grade deionized water (ASTM Type I water), was added to each tube to bring the total solution volume to 10.0 ml.

5. Prior to analysis, each sample was filtered through a 0.45 μm membrane syringe filter.

Analysis of the sample digests was conducted on a Perkin Elmer Optima 3000 DV ICP Emission Spectrometer. This instrument, equipped with an autosampler and integral computer workstation, is configured to detect up to 30 elements simultaneously in less than five milliliters (5 ml) of sample solution. The principle of analysis is the detection of characteristic ultraviolet and visible light emissions from metallic elements subjected to a high temperature argon plasma torch. To improve instrument performance, a Yttrium internal standard is added to each sample, standard and blank to compensate for small variations in sample flow rate, sample viscosity and acid concentration and to assist in the identification of potential interferences. Quantification of the metallic analytes in the sample is based on the measurement of specific wavelength intensities for each element and comparison of these results to multi-point calibration standards analyzed in the same manner.

The instrument is calibrated daily with three multi-component standards and a blank. A series of verification standards, interference check solutions and blanks are analyzed and evaluated before any samples are analyzed. At a frequency of every ten samples, a calibration verification standard and blank are analyzed. Acceptance criteria for each standard, blank and sample measurement are defined and used to accept or reject results.

Discussion

The elemental analyses for zygotic embryo are very similar for the five seed sources tested. Summaries of the averages per seed source and for all replications are shown for zygotic embryo in Table 1. Analyses for female gametophyte tissue is also similar for the five seed sources and is shown in Table 2. Standard errors for zygotic embryo elemental variation between all of the replicates for Mn, Fe, Cu, Zn, P, S, Mg, and K are less than 4% of the mean values (Table 1). Nickel, B, Na, and Ca show greater variation with standard errors ranging from 5-16% of the mean (Table 1). Standard errors for female gametophyte elemental variation between all replicates for Fe, Ni, Cu, Zn, P, S, Mg, and K are less than 4% of the mean values (Table 2.). Manganese, B, Na, and Ca show greater variation with standard errors ranging from 5-8%.

Individual replicate analyses for zygotic embryos per site are shown in Tables 3-7. Individual replicate analyses for female gametophyte tissue per site are shown in Tables 8-12. Elemental concentrations detected for Cobalt, Nickel, Molybdenum, and Sodium were sometimes below the accurate detection limits of the equipment and values for these replicates are shown as <.

Elemental concentrations of zygotic embryo and female gametophyte tissues were often different. Ratios of elemental compositions, on a dry weight basis, are shown in Table 13.

Embryos contained low contents of Mn, B, and S, suggesting that these elements are selectively excluded from the embryo. Similar contents of Ca, Ni, Zn, and Cu were found in embryo and female gametophyte tissue suggesting that these are taken into the embryo by diffusion. Greater concentrations of P, K, Mg, Na, and Fe were found in the embryo compared to female gametophyte tissue suggesting that these elements are actively taken up by the embryo.

Overall, the similarity in analyses of zygotic embryo tissues suggest that the mean elemental compositions of zygotic embryos provide reasonable targets for the elemental composition of somatic embryos.

Table 1. Summary of averages of replicated elemental analysis of zygotic embryos collected from loblolly pine seeds grown on different mother trees and in different locations.

Tree	Location	Reps	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
BC-1	Lake Charles, LA	5	89.4	258		3.4	28.7	146	6.0	17053	2747			7671	12678	173
UC5-1036	Bellville, GA	4	90.6	279		1.0	24.6	133	8.7	17432	2619			8122	13092	275
7-56	Summerville, SC	3	66.4	221		1.0	22.7	113	1.2	15440	2242		7.1	7352	11552	140
UC10-14	Rincon, GA	2	76.7	181			31.0	113	2.2	14883	2312		6.6	7283	11219	174
UC10-1003	Rincon, GA	1	82.1	215	<.40	<.59	30.0	147	<.36	16421	2410	<.52	<3.23	7618	11833	146
Mean		15	83.0	243	####	2.1	26.8	132	6.2	16500	2531	####	6.6	7672	12312	192
Std Error		15	3.0	9.7	####	0.3	0.8	4.3	1.0	282	57	####	0.3	99	213	28
Std Er/Mean			0.04	0.04	####	0.16	0.03	0.03	0.16	0.02	0.02	####	0.05	0.01	0.02	0.15

Table 2. Summary of averages of replicated elemental analysis of female gametophyte tissue embryos collected from loblolly pine seeds grown on different mother trees and in different locations.

Tree	Location	Reps	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
BC-1	Lake Charles, LA	5	243	74		3	20	127	28	12415	5867			5019	9410	203
UC5-1036	Bellville, GA	4	233	82			22	143	21	13525	5547			5453	9744	344
7-56	Summerville, SC	3	121	53			16	126	17	10804	4633		4	4621	7843	270
UC10-14	Rincon, GA	2	251	79			18	155	19	11981	5430		4	5435	9102	150
UC10-1003	Rincon, GA	1	287	66	<.35	<.55	21	193	12	12348	5465	<.45	<2.83	5129	9460	311
Mean		15	221	72	####	2.6	20	139	22	12327	5450	####	3.9	5118	9148	254
Std Error		15	14.8	3.1	####	0.1	0.6	4.9	1.5	256	120	####	0.2	88	199	21
Std Er/Mean			0.07	0.04	####	0.03	0.03	0.04	0.07	0.02	0.02	####	0.05	0.02	0.02	0.08

Table 3. Elemental analysis (mg/Kg) for zygotic embryos of BC-1 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
BC-1	89.3	283.4	<0.43	3.5	29.2	154.7	7.7	17302	2715	<0.55	<3.44	7740	12675	171.9
BC-1	87.9	249.8	<0.43	4.1	28.9	139.5	6.9	16806	2711	<0.55	<3.45	7617	12708	200.7
BC-1	89.1	271.3	<0.40	2.3	30.4	160.6	4.2	17543	2824	<0.52	<3.24	7924	13009	137.0
BC-1	97.8	266.3	<0.40	3.3	28.4	147.0	3.1	17525	2793	<0.51	<3.22	7823	13050	180.2
BC-1	83.2	218.3	<0.42	3.9	26.7	129.5	8.1	16091	2692	<0.54	<3.40	7253	11948	175.6
Mean	89.4	257.8		3.4	28.7	146.3	6.0	17053	2747			7671	12678	173.1
Std Error	2.4	11.3		0.3	0.6	5.5	1.0	274.9	25.8			116.1	197.8	10.3

Table 4. Elemental analysis (mg/Kg) for zygotic embryos of Union Camp 5-1036 from full term cones collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
UC5-1036	83.2	267.3	<0.42	1.4	24.6	129.4	8.0	16961	2539	<0.54	<3.35	7958	12791	150.6
UC5-1036	97.6	291.6	<0.42	0.9	24.6	126.6	3.1	17630	2652	<0.54	<3.39	8158	13196	188.9
UC5-1036	100.6	292.7	<0.38	0.7	25.2	143.7	14.3	17700	2595	<0.48	5.1	8273	13120	574.1
UC5-1036	81.2	265.5	<0.42	1.1	24.0	130.5	9.3	17435	2688	<0.54	<3.37	8098	13259	185.1
Mean	90.6	279.3		1.0	24.6	132.5	8.7	17432	2619			8122	13092	274.7
Std Er	4.9	7.4		0.2	0.2	3.8	2.3	167	33			66	104	100.2

Table 5. Elemental analysis (mg/Kg) for zygotic embryos of Westvaco 7-56 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
7-56	73.0	227.6	<0.40	1.4	24.8	118.3	1.2	16132	2340	<0.51	8.4	7707	12232	100.7
7-56	60.6	210.5	<0.38	0.7	21.5	108.9	<0.35	14617	2122	<0.49	7.2	6948	10732	115.4
7-56	65.8	223.8	<0.41	<0.61	21.9	112.5	<0.38	15572	2265	<0.53	5.8	7401	11691	203.9
Mean	66.4	220.7		1.0	22.7	113.2	1.2	15440	2242		7.1	7352	11552	140.0
Std Er	3.6	5.2		0.3	1.1	2.7	0.0	442	64		0.7	220	438	32.2

Table 6. Elemental analysis (mg/Kg) for zygotic embryos of Union Camp 10-14 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
UC10-14	73.5	179.6	<0.40	<0.58	31.2	115.3	<0.36	15063	2312	<0.51	<3.18	7338	11328	157.1
UC10-14	79.9	181.5	<0.41	<0.60	30.8	110.5	2.2	14703	2312	<0.53	6.6	7228	11109	191.8
Mean	76.7	180.6			31.0	112.9	2.2	14883	2312		6.6	7283	11219	174.5

Table 7. Elemental analysis (mg/Kg) for zygotic embryos of Union Camp 10-1003 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
UC10-1003	82.1	214.8	<0.40	<0.59	30.0	147.5	<0.36	16421	2410	<0.52	<3.23	7618	11833	146.5

Table 8. Elemental analysis (mg/Kg) for female gametophyte tissue of BC-1 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
BC-1	237.2	78.5	<0.37	2.2	21.2	129.2	28.3	12624	5774	<0.48	<3.00	4934	9503	240.8
BC-1	264.1	70.5	<0.38	2.8	19.5	131.8	23.3	12291	5883	<0.49	<3.06	4785	9343	198.6
BC-1	248.2	79.8	<0.38	2.3	19.8	129.4	31.2	12619	5775	<0.49	5.4	5128	9530	208.0
BC-1	257.3	77.3	<0.41	2.7	21.2	130.5	28.0	12636	5977	<0.52	<3.26	5199	9943	111.7
BC-1	205.8	65.6	<0.40	3.1	18.6	114.7	28.1	11907	5925	<0.51	<3.22	5050	8732	257.9
Mean	242.5	74.4		2.6	20.1	127.1	27.8	12415	5867			5019	9410	203.4
Std Er	10.2	2.7		0.2	0.5	3.1	1.3	143	41			73	197	25.3

Table 9. Elemental analysis (mg/Kg) for female gametophyte tissue from Union Camp 5-1036 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
UC5-1036	231.8	72.9	<0.40	<0.60	22.2	141.5	16.1	13448	5606	<0.52	<3.25	5455	9761	294.5
UC5-1036	241.5	88.4	<0.39	<0.58	22.2	151.9	20.8	13944	5600	<0.50	<3.14	5600	10024	402.2
UC5-1036	248.5	86.1	<0.39	<0.57	22.3	138.2	22.7	13218	5537	<0.50	<3.11	5278	9449	292.0
UC5-1036	211.7	79.2	<0.41	<0.60	21.2	141.2	22.9	13493	5444	<0.52	3.8	5477	9743	386.5
Mean	233.3	81.7			21.9	143.2	20.6	13525	5547			5453	9744	343.8
Std Er	8.0	3.5			0.3	3.0	1.6	152	38			66	118	29.4

Table 10. Elemental analysis (mg/Kg) for female gametophyte tissue of Westvaco 7-56 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
7-56	145.6	50.3	<0.34	<0.51	16.9	126.8	19.9	10913	4638	<0.44	3.9	4657	8150	253.5
7-56	100.0	51.1	<0.33	<0.48	15.4	120.5	16.1	10561	4451	<0.42	3.4	4570	7568	273.3
7-56	118.8	56.9	<0.33	<0.49	16.6	129.9	15.6	10936	4811	<0.42	3.3	4636	7811	284.3
Mean	121.4	52.7			16.3	125.7	17.2	10804	4633		3.5	4621	7843	270.3
Std Er	13.2	2.1			0.5	2.7	1.3	121	104		0.2	26	169	9.0

Table 11. Elemental analysis (mg/Kg) for female gametophyte tissue of Union Camp 10-14 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
UC10-14	210.5	80.3	<0.37	<0.54	17.4	157.8	17.7	11846	5472	<0.47	3.0	5381	8792	149.1
UC10-14	291.4	78.4	<0.39	<0.58	18.5	152.4	20.3	12116	5388	<0.50	4.3	5490	9412	150.6
Mean	251.0	79.4			17.9	155.1	19.0	11981	5430		3.6	5435	9102	149.9

Table 12. Elemental analysis (mg/Kg) for female gametophyte tissue of Union Camp 10-1003 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
UC10-1003	287.0	66.4	<0.35	<0.55	21.3	192.7	11.6	12348	5465	<0.45	<2.83	5129	9460	310.7

Table 13. Ratio of average elemental concentrations in dried zygotic embryo vs. female gametophyte tissues.

Ratio	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
Zyg/FG	0.38	3.37	#####	0.80	1.37	0.95	0.29	1.34	0.46	#####	1.72	1.50	1.35	0.75

Mass Clonal Propagation of Improved Conifers: Antibody Markers, Staining Procedures and Image Analysis for Characterizing *Pinus taeda* Embryogenesis

Gary Peter
Levi Barclay

Summary: Antibodies are sensitive markers for determining the presence of specific proteins. We propose to develop rapid methods for using antibodies to stain single embryos. We are searching for antibody markers that can be used to determine the developmental stage of single embryos and more importantly ones that predict the developmental potential of somatic embryos. For example, we have been testing for shoot apical meristem formation with an antibody which recognizes the Knotted and Rough Sheath, homebox containing proteins from maize. We can also determine how many embryos are in a given developmental stage and evaluate the synchronization of embryos in our cultures. The practical use of such predictive markers when combined with image analysis will help us to objectively evaluate embryo development as well as to determine earlier and with more certainty the developmental potential of individual embryos in our cultures. These markers will be used as one of our guides for optimizing culture conditions to improve embryo quality and embryo maturation and to investigate culture cycling as well as embryo culture decline.

Goals:
1. Develop standard procedures for fixation and whole mount staining of embryos with antibodies.
2. Initiate screening for antibodies that react with staged embryos and preceed critical transitions.
3. Determine whether antibodies are predictive of developmental potential.
4. Use antibodies and image analysis to characterize synchrony of embryos in cultures.

Materials: *Stages:* 2, 3 and 9.8, *Genotypes:* BC-1, UC5-1036, *Zygotic Embryo Collection*: 1996 and Somatic embryos

Solutions: 1X phosphate buffered saline (PBS) 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM Na H_2PO_4, pH 7.0; 1X Tris buffered saline (TBS) 130 mM NaCl, 10 mM Tris-HCL, pH 7.0; milk protein is nonfat dried milk powder we have used the brand name Carnation

Equipment: Zeiss Axioplan 2 equipped with differential interference contrast and UV fluorescence capabilities. Sony DKC-5000 integrating, three chip color camera for acquiring digital images directly onto the computer for image analysis.

Methods:

Fixation: The best results for fixing somatic embryos were obtained by adding formaldehyde to a final concentration of 3.7% by diluting stock 37% formaldehyde directly into somatic embryo growth media. The fixation was carried out for 1 hour at room temperature and then 4^0C overnight. Zygotic embryos were also fixed in 3.7% formaldehyde overnight at 4^0C. The addition

of 0.25% glutaraldehyde was found to help preserve integrity of embryos for embedding into paraffin wax.

Antibody Staining: The primary antibodies are incubated at room temperature or 37°C for 1-12h with the embryos in a small volume of 1XPBS + 3% MP at an appropriate dilution. The embryos are then washed extensively (4X) with 1XPBS + 3% MP, for 30 minutes. Secondary antibody binding is carried out as described above for primary antisera. Secondary antibodies are specific to the source of primary antibody and are conjugated either to an enzyme or to fluorescent dyes. In plants chromogenic detection, typically uses alkaline phosphatase conjugated to antibodies since high levels of endogenous peroxidase activity limit the use of this enzyme for histochemical staining. For fluorescence detection, fluorescein (FITC) and Cy-5 are the best dyes because these excitation and emission wavelengths give the least interference from background auto-fluorescence. To clear the tissue for viewing it is resuspended in chloral hydrate of neutral pH.

Wholemount Pretreatment: To facilitate up take of antibodies the membranes were permeabilized with 0.1% Tween 20 for 30 minutes in antibody blocking solution, 1X PBS + 3% milk protein (MP). The high protein content helps block sites within the tissues that might non-selectively bind antibodies. The detergent was removed with a subsequent 1X PBS + 3% MP wash step. An additional step of partial cell wall hydrolysis can also facilitate antibody uptake; however, the pores of the walls do not limit diffusion of the antibodies.

Rationale of this Approach:

This work aims to develop rapid antibody staining procedures, to identify antibodies that define specific developmental stages, and to identify antibodies that can be used to predict the developmental potential of somatic embryos. We would like to integrate our current morphological staging system with a complementary biochemical and developmental based staging system. Developing such a system will benefit both our tissue culture experiments and our differential display studies. One primary benefit is to assay earlier and more objectively populations of embryos for their developmental response to our culture conditions. A second benefit is to begin correlating gene expression with the stage of development in a more precise way. Another is to begin investigating the synchrony of embryo development in our cultures. The synchrony of embryo maturation is an important practical goal since we would like most of the embryos to mature and to germinate within a reasonably similar time for commercial production of trees. It may also be important for improving embryo quality since growing embryos communicate as well as compete both through promotive and inhibitory mechanisms.

Progress:

Image Acquisition and Analysis: We have recently purchased a Zeiss Axioplan 2 equipped with differential interference contrast and UV fluorescence capabilities. We have also purchased a Sony DKC-5000 integrating, three chip color camera for acquiring digital images directly onto the computer for image analysis. We are using multiple different image analysis software, including Photoshop, NIH Image, and Optumus. With this equipment we can obtain high quality images directly onto the computer for image analysis and quantitation.

Antibodies: The first antibody that we have concentrated on is one that was raised against the Knotted/Rough Sheath proteins, which are homeobox containing proteins, that are shoot apical meristem and subapical meristem specific in *Zea mays* (1,2). They are also expressed in the embryo and mutations in them are cause an embryo lethal phenotype (3,4). Secondary antibody conjugates, anti-rabbit and anti-mouse alkaline phosphatase, and FITC were obtained from Boerhinger Manheim. This secondary antibodies are of immunohistology grade.

Approach to Standardize Method: We have been testing the antibodies for their reaction with formaldehyde fixed mature pine embryos embedded in paraffin wax, sectioned to 8 uM thickness and applied to Probe On Plus Slides. After deparaffinizing, the sections were blocked and treated with antibodies as described in the methods section. The results of these studies will be presented.

References:

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2. Schneeberger, R. G., Becraft, P. W., Hake, S. and Freeling, M. (1995) Ectopic Expression of the Knox Homeo Box Gene Rough-Sheath-1 Alters Cell Fate in the Maize Leaf. Genes & Development: 9 (22) p.2870-2880.

3. Schneeberger, R. G. and Freeling, M. pers. comm.

4. Smith, L. G. and Hake, S. pers. comm.

Mass Clonal Propagation of Improved Conifers: Construction of cDNA Libraries from Zygotic Embryos of *Pinus taeda*

Gary Peter
Gerald Pullman

Summary: One basic approach for improving somatic embryos is to understand how they differ from zygotic embryos. To analyze these differences at the molecular genetic level we need to construct cDNA libraries that represent all of the mRNAs expressed in embryos at the various stages. Due to the limiting tissue, especially in the early stages of embryogenesis, this task is made difficult by the requirement for large amounts of RNA for traditional cDNA library construction methods. To overcome this limitation, we have applied the CAP-Switch methodology from Clontech to make representative, full-length cDNA libraries in λ-gt11 from small amounts of total RNA. With this method we have synthesized mostly full length cDNAs from small amounts (as little as 200 ng) of total RNA isolated from staged *Pinus taeda* zygotic embryos. We are presently using this cDNA to construct libraries. We are also testing whether this cDNA can be used as pseudo mRNA for direct analyses of gene expression from zygotic embryos. These libraries will provide a resource for isolating full length cDNAs from genes identified by the differential display efforts and by antibody screening with antiserums identified from antibody marker approaches.

Goals:

1. Construct libraries that have a high proportion of full length cDNAs and that represent all of the mRNAs that are expressed in stage 3, 4, 8, 9.2 and 9.8 zygotic embryos of *Pinus taeda.*
2. Test whether CAP-Switch is a valid method to prepare pseudo mRNA from small amounts of total RNA for direct analysis of mRNA levels.
3. Provide a resource for isolating full length cDNAs from zygotic embryos to support efforts from differential display and antibody screening.

Materials: *Stages:* 3,4,8, 9.2 and 9.8 *Genotypes:* UC5-1036, BC-1 *Zygotic Embryo Collection:* 1996

Results and Discussion:

RNA Extractions: The Tri-Reagent protocol was followed with LiCl precipitation for stages 8, 9.2 and 9.8. Tri-Reagent is based upon the guanidium acid phenol method (1). The acidic phenol of this method effectively removes most of the DNA from the RNA samples. Stage 3 and 4 embryos were so small a quantity that the extraction was performed in 200 ul of extraction buffer and no LiCl precipitation was carried out. In these preparations, extra attention was paid not to get any interface while pipeting off the supernatant, which contains the RNA, from the phenol phase, which contains the DNA. RNA isolated from stages 8 and 9.2 were extracted in 0.5 ml of buffer; whereas, 9.8 embryos were extracted with 2 ml of buffer. To remove contaminating charbohydrates stage 8, 9.2 and 9.8 RNAs were precipitated with 2.0 M LiCl.

TABLE 1: Isolation of RNA

Stage	Total RNA (ug)	Est. FW/20 E (mg)*	# embryos	RNA/embryo (ug)
3	0.5	1.20	20	0.025
4	5.0	1.56	20	0.25
8	6.15	10.39	20	0.325
9.2	7.2	31.90	20	0.36
9.8	100.0	45.35	50	2.00

*Estimates derived from 1994 embryos collections. Gerald Pullman, unpublished

First Strand cDNA Synthesis: These steps were carried out according to the CAP-Switch protocol. The initial synthesis was done with MMLV reverse transcriptase and the corresponding amount of RNA presented in Table 2.

TABLE 2: Actual # of Cycles

Stage	Starting RNA (ug)	# of cycles
3	0.2	22
4	2.0	20
8	2.0	14
9.2	2.0	20
9.8	2.0	14

Second Strand cDNA Synthesis and PCR Amplification: After first strand cDNA synthesis, PCR amplification of the resulting cDNA was performed to synthesize the second strand and to increase the amount of cDNA for subsequent cloning with the long-range PCR reagents supplied. The suggested number of cycles to be carried out depends on the starting amount of RNA as shown in Table 3.

TABLE 3: Suggested # of Cycles*

Total RNA (ug)	# of Cycles
1.0-2.0	18-20
0.5-1.0	20-22
0.25-0.5	22-24
0.05-0.25	24-26

*From CAP-Switch Protocol, Clontech

This amplification step is critical to the performance of the approach and needs to be optimized for each RNA. Ideally a smear of cDNAs with an average size of ~1.6 kb for plant mRNAs and still with some prominent discrete bands should be observed. As can be seen from Table 3, Figures 1 and 2, the number of cycles needs to be determined empirically.

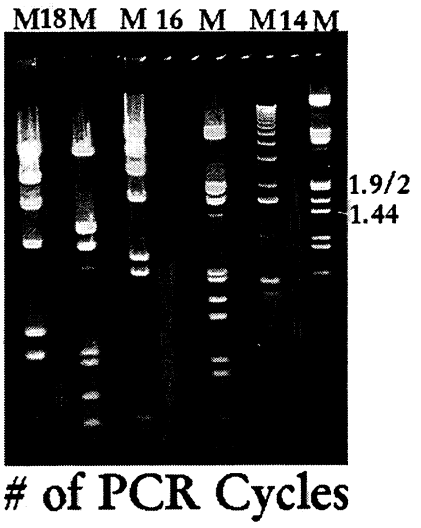


Figure 1. The effect of cycle # on average size and ability to see individual bands

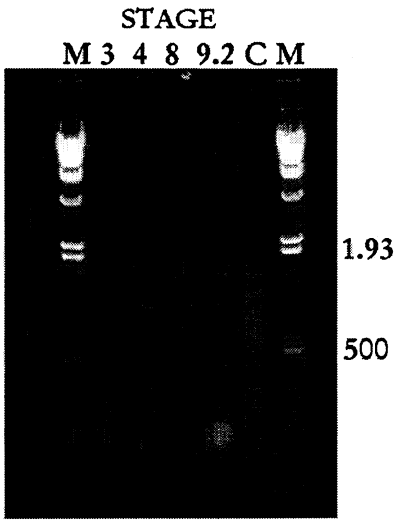


Figure 2. Amplification of cDNAs from zygotic embryos

The strategy that worked the best for us was to perform 4 cycles less than the lowest number suggested and then evaluate the products. If not enough product has been synthesized then additional cycles can be preformed with the same reaction. It is very important not to over amplify the cDNA to maintain a better representation of the starting mRNA population.

Size Fractionating of cDNA for Library Construction: The cDNA was passed through a gel filtration column to separate the cDNA by size. The average size of the cDNA in each fraction was determined by gel electrophoresis, Figure 3. Fractions containing cDNAs > 400-500 base pairs were pooled and precipitated.

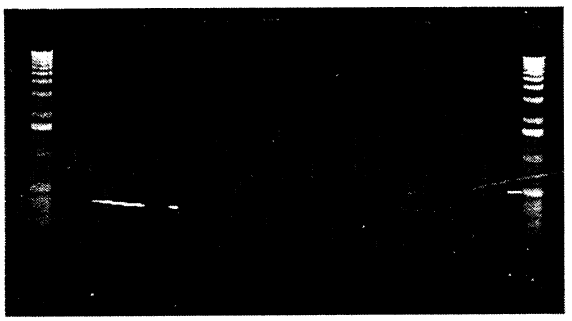


Figure 3. 3 ul of each ~35 ul fraction was separated by agarose gel electrophoresis.

Ligation to λ-gt11 Arms and in vitro *Packaging*: The recovered, size fractionated cDNA was ligated to the lambda arms provided in kit. The ratio of cDNA to arms was varied. The reactions were packaged in vitro with GigaPak III Gold from Stratagene.

Characterizing the Quality of cDNA Libraries: cDNA library quality is assessed by the number of total phage, the percent of phage that have cDNA inserts, recombinant phage, and the average size of the cDNA inserts. A good quality library should have at least 1-2 x 10^6 phage with at least 75% having an insert. The total number of packaged phage are determined by plating on a permissive host. The number of phage that have cDNA inserts are determined simply by growth on Y1090 *E. coli*, in the presence of isopropyl-β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal); blue plaques do not have an insert while white plaques have an insert, due to the interruption of the β-galactosidase reading frame. The average size of inserts are determined by amplifying white plaques with primers that flank the cDNA cloning site and separating the PCR products by agarose gel electrophoresis. For efficient PCR of phage picks, the agar plugs with phage are placed in 100 ul of water and incubated for 30 min with shaking. Then 10 ul of this stock is taken and submitted to 3 freeze/thaw cycles. The characterizations of the libraries will be presented in the PAC meeting.

Anticipated benefits of high quality cDNA libraries from zygotic embryos of *Pinus taeda*:

- These libraries will help us to identify differences in gene expression between somatic and zygotic embryos of the same morphological stage. This comparison provides a critical guide for defining the developmental status and potential of somatic embryos relative to zygotic embryos and for discovering predictive molecular assays to help us improve our culture conditions that enhance embryo quality.

- These libraries provide a resource to isolate genes, both by antibody screening and low stringency hybridization, identified to be important for embryogenesis in crop and model plants.

- These libraries can be used as a principle resource for identifying stage specific cDNAs from zygotic embryos through subtractive hybridizations and/or differential screening.

- These libraries can be screened to isolate full length clones that correspond to cDNAs obtained from the differential display efforts. Full length cDNA clones will provide additional sequence information needed to help determine the possible function of these proteins. In addition, full length cDNAs will be used for generating high titer antibodies to differentially expressed genes. These antibodies will facilitate single embryo assays with whole mount staining procedures.

- The original cDNA may be a source of pseudo mRNA that can be used for screening clones for differential expression in different staged zygotic RNA populations in reverse northern style experiments and possibly for sizing of zygotic mRNAs.

- If antibodies are identified through screening procedures that are predictive markers of embryo development under our culture conditions, then cDNAs that encode these proteins can be isolated by antibody screening.

- These libraries can be used as a source of cDNAs for random sequencing efforts being carried out in *Pinus taeda.*

References:

1. P. Chomczynski & N. Sacchi (1987) Single-step Method of RNA Isolation by Guanidinium Thiocyanate-Phenol-Chloroform Extraction. *Anal. Biochem.*: **162** 156-159.

Differential Display as a Tool to Understand and Improve Somatic Embryogenesis

John Cairney

Differential Display has the potential for allowing us to follow gene expression over the course of embryogenesis. Our goal over the past two years has been to apply this technique to a few genotypes of pine embryos, with a view to extending it to many genotypes and further, to compare gene expression in somatic and zygotic embryos.

The scheme of these studies, and a summary of the information in the reports, is given below.

1. Demonstrate a reproducible banding pattern (gene expression). Results reported in the last PAC report and meeting (October 1996), show that banding patterns can be confirmed by running duplicate samples and by using several starting RNA concentrations. New, rapid and accurate analysis using an oligo-dT bead-primed method is accelerating and improving our efforts.
2. Identify bands which are diagnostic of a phase or stage of development. As more embryos of different stages and genotypes have been isolated and used for DD, bands of each of the aforementioned categories have been identified.
3. Use the sequence to extend the usefulness of the markers. Those clones which appear most useful in characterizing a stage of development (reliably observed, easy to spot) can be employed to analyze several genotypes. *Long, specific primers* which can be designed, based on the cDNA sequence (see 4-6), and then be used in RT-PCR. Such Long Primers, 20-mers for example, should be able to hybridize to homologous RNA/cDNA from many sources despite occasional mismatches. An alternative strategy would involve employing cloned sequences in direct expression analysis, by methods such as RNase protection.
4. Clone these bands. At the time of writing approximately 40 bands have been excised from gels and cloned. These are being sifted by restriction analysis.
5. Characterize their expression. Those cDNAs initially responsible for the differential expression observed are being identified by large scale screening methods and will subsequently be subjected to more refined expression analysis
6. Sequence these partial cDNAs. The differentially expressed cDNAs will be sequenced and compared to sequences in the GenBank. Where we have positive identifications, we will then be able to relate the appearance of a certain mRNA (and by implication, protein) to a certain stage in embryo development. This type of correlation will allow many inferences to be made about embryo development. These inferences can be developed into testable hypotheses which will lead ultimately to growth culture modification.
7. Repeat experiments with zygotic embryos. Differential Display reactions have been conducted with zygotic embryos using the oligo-dT bead-primed method and clear banding patterns have been identified. Several bands have been identified and cloned.

We will pursue these as outlined above and will further compare expression of genes identified as being expressed in somatic embryos at different stages and determine whether they follow a similar pattern in zygotic embryos

8. Isolate longer clones. We will, in certain cases, isolate 'full-length' or at least longer versions of these genes from a cDNA library constructed from zygotic embryo RNA (see section by Gary Peter). These may then be used to generate proteins and from there antibodies can be generated which will be used to follow protein markers.
9. Determine where, how and why these genes switch on. We will seek to identify factors (hormonal, cultural) which induce or repress gene expression and determine where, within the embryo, these genes are expressed.
10. The nature of culture cycling will be probed using these tools and techniques, addressing a problem which troubles many labs.

Monitoring Gene Expression in Somatic Embryos of an Elite Genotype of Loblolly Pine

John Cairney and Barbara Johns

Total RNA of stages 4-9 has been extracted from genotype 260 using TRI Reagent® Isolation Reagent (Molecular Research Center, Inc.), and characterized by differential display technique. TRI Reagent® includes phenol and guanidine thiocyanate in a mono-phase solution. It has the advantage of being time-efficient and easy to use, isolating quality RNA from a small amount of tissue (80-100 mg). However the interference in spectrophotometric readings by polysaccharides can sometimes make quantification difficult. This can be overcome by comparing known quantities of RNA with the sample RNA on a 1% agarose gel.

Embryos are homogenized in TRI Reagent® and the homogenate is separated into the aqueous phase and organic phases by the addition of bromochloropropane and centrifugation. The RNA stays exclusively in the aqueous phase and is precipitated with isopropanol, washed with 75% ethanol, and resuspended in sterile water.

RNA yield is quite variable between stages but adequate amounts of good quality RNA (48-75 μg) have been isolated from later stage embryos (7-9). Earlier stage embryos have a lower RNA content in comparison to the weight of tissue homogenized. Suspensor tissue may account for as much as 90% of this weight. These cells have a low physiological activity and are almost devoid of RNA. This was reflected in the yield of RNA extracted from stages 4-6 (7-14 μg).

RNA Yield by Embryo Stage of Genotype 260

Embryo stage	Amt. tissue(mg)	Amt. RNA(μg)	RNA yield(μg/g)
4	81	7	95
5	65	13	214
6	99	14	197
7	79	48	611
8	97	75	780
9	101	65	652

After conversion of the RNA in the presence of the restriction enzyme MMLV (RT-PCR), the cDNA was amplified with AmpliTaq polymerase and α ^{35}S dATP. As a check three dilutions of 0.1, 0.25, and 0.75 μg were used. The samples were electrophroesed on

a 6% acrylamide gel and visualized on autoradiograph film. Several bands were observed and selected for cloning.

Total RNA isolated by this method has been used for differential display and distinctive banding patterns observed. The patterns will be compared with those generated with RNA from different genotypes.

DIFFERENTIAL DISPLAY TO COMPARE GENE EXPRESSION IN LOBLOLLY PINE ZYGOTIC AND SOMATIC EMBRYOS

Vincent Ciavatta, Jerry Pullman and John Cairney

Project Goals:

1. The primary goal is to uncover differences in gene expression (1) between analogous stages of somatic and zygotic embryos and (2) between embryos from different stages of development.

2. It is a further goal of this research to determine how the genes found through differential display play a role in successful embryogenesis and use this knowledge to augment somatic embryogenesis technology.

Project Update:

Somatic culture #255 has been maintained in liquid suspension. Periodically, aliquots of this culture are flash frozen in liquid N_2 while a separate aliquot is plated-out on maturation and development medium. Somatic embryos are grown in this fashion to all of the various stages of development. At some point, embryos of a desired stage are plucked from culture, flash frozen in liquid N_2 to preserve the RNA, and stored at -70°C until the RNA is isolated. Renewability of somatic embryos makes them a good source of RNA. As a consequence differential display work thus far has been conducted exclusively with somatic embryos in order to:

- Work through problems involved with differential display (e.g., RNA isolation, repeatability, etc.)
- Produce a profile of gene expression during embryo maturation so that candidate differentially expressed sequences can be identified

RNA Isolation & Repeatability

Originally, RNA for differential display was isolated by a method that was developed specifically for pine tissue (Chang et al. 1993. Plant Mol. Biol. Reporter 11: 113-116). This method requires an overnight precipitation so a quicker method was sought. Some success has been had with Tri Reagent (Chomczynski et al. 1993. BioTechniques 15: 532-537). Use of Tri Reagent results in RNA yield (by spectrophotometry) and RNA quality (by RNA gel and A_{260}/A_{280}) that are comparable to those measures of Chang et al.-isolated RNA. More importantly, differential displays from the same RNA pool have proven to be very similar regardless of the method of RNA isolation. Additionally, Tri Reagent-isolated RNA has produced the cleaner differential displays which facilitates gel reading and band excision.

Differential display banding patterns are checked for repeatability by conducting two separate PCR reactions for each RT reaction. This is done to avoid the possibility of selecting a false positive band that may have arisen via incorrect priming of the template DNA or a contaminating sequence. Checking that a potentially stage-specific band is produced from both PCR reactions can limit time wasted investigating (i.e., excising, amplifying, and cloning) a false positive band.

Somatic Embryo Gene Expression Profile

Using RNA isolated by both the Tri Reagent (Figure 1) and Chang et al. methods, partial profiles of gene expression (differential displays) were generated. In hopes of identifying real differentially expressed sequences, 10 bands were excised from the differential display gels and then carried through the following:

- Amplification and ligation into the pCR®2.1 vector
- Competent cells transformed with sequence-containing plasmids
- *Colony PCR*: 8 to 10 colonies per plate selected and checked for inserts via PCR amplification with primers specific to flanking sequences of the pCR®2.1 insert
- *Restriction Enzyme Fingerprinting*: determine which colonies have the same sequence
- Confirmation of differential expression (potentially accomplished by several methods)

Bands which are excised from differential display gels usually contain several types of co-migrating DNA. Only one of these will be responsible for the different expression pattern observed on the X-ray film, in the simplest case, therefore it is important to determine how many classes of sequence have been cloned and which of these is the clone of a developmentally expressed gene. When the cDNAs of these clones are digested with restriction enzymes which have a 4 base pair recognition sequence, several classes of can be distinguished. The original ten bands excised at the start of this process have been shown to be comprised of 25+ classes of sequences. This would suggest that each band excised from the initial differential display gel is heterogeneous (contains 2 to 3 different sequences). This finding, as stated above, is not unique as it has been noted by others (Shogham et al. 1996. BioTechniques 20(2): 182-183).

At the time of writing, these 25 classes of sequences have not yet been confirmed for differential expression. Potential methods for this task are:

- *Northern Blotting*: This process requires several microgram of total RNA and may be possible for liquid suspension culture or late stage bands where obtaining large amounts of RNA may be feasible
- *Dot* or *Slot Blotting*: is a useful technique for analyzing large numbers of clones simultaneously and serves as a tool to identify developmentally regulated clones from among 'false positives' by comparing expression at two stages of development.

Large amounts of RNA may be needed or, alternatively, labeled cDNA libraries can be used

- *RNase Protection Assay*: potentially useful for assaying the level of expression of a sequence that exhibits a changing level of expression on the initial differential display profile. This is the most sensitive method requiring little RNA. Additionally multiple probes can be used simultaneously with the same valuable RNA.

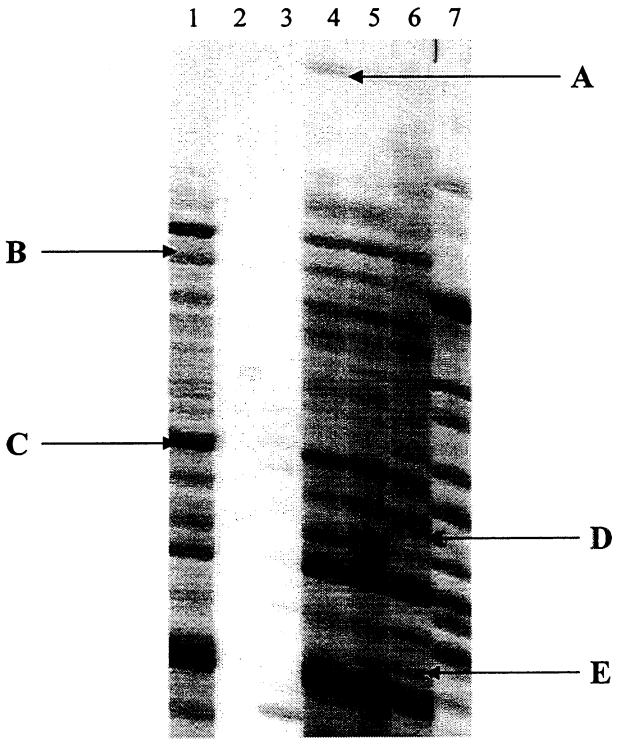


Figure 1. An autoradiogram of a differential display gel. RNA for this gel was isolated with Tri Reagent, reverse transcribed with $T_{12}MC$, and amplified with arbitrary primer #4 (AP-4). Lanes 2 and 3 were not loaded with the correct PCR products which accounts for the aberrant banding patterns. PCR products in lanes 1, 4, 5, and 6 were generated from stages 4, 7, 8, and late stage (9) embryos, respectively, and lane 7 is a control generated from mouse RNA. Bands indicated by arrows A, B, C, D, and E were excised from the gel and are currently being characterized. Bands B and C were strongest at stage 4 and diminish with embryo growth. Band A was absent at stage 4, strongest at stage 5 (not shown), and diminishes with embryo growth. Band D appears exclusive to late stage embryos. Finally, Band E is faintly present at stage seven and increases to a maximum at late stage.

Future Goals

1. Current bands under investigation that are confirmed to exhibit differential expression will be sequenced and compared with other Genebank sequences. Another gene expression profile of somatic embryos will be generated from poly-A RNA (see #2 below) and characterize more differentially expressed bands.

2. Because comparison of zygotic and somatic embryos is the ultimate goal of the project, work will soon commence to generate a gene expression profile of zygotic embryos that were collected last summer. Because zygotic tissue is limiting, a different, potentially more sensitive method of RNA isolation may be used. This method, which isolates only poly-A RNA is currently being used by Nanfei Xu.

Analysis of Somatic Embryo Development Using Differential Display: Rapid Identification, Cloning and Sequencing of Marker Bands by an Improved Method.

Nanfei Xu, Jerry Pullman, and John Cairney

Previous Achievement:

1. Characteristic band patterns were observed in differential display using large quantities of total RNA extracted from needles, stems and roots of loblolly pine.
2. Different differential display patterns were obtained for early stage somatic embryos derived from different genotypes.

New Development:

Solid-phase RNA differential display was optimized for loblolly pine embryos.

Differential display (DD) can be performed using total RNA. It is easy to isolate sufficient amounts of RNA for DD if the quantity of embryo material is abundant, however with early stage embryos the quantity of starting material is very limited and loss of RNA which occurs during its isolation by conventional methods, including Chang et al and TRI-Reagent methods described earlier, would render such methods impractical for early-stage embryos. With Somatic Embryos, the continual supply of embryos of different stages allows us to increase quantities of starting material and thus overcome the problem, however, where embryos of a specific stage are limiting, such as when working with zygotic embryos, this approach cannot be employed.

To overcome these problem, we used coated magnetic beads with attached oligo(dT) to isolated poly(A) RNA. In this method, the embryos (5-10 mg) were ground in a lysis buffer and the clarified lysate was added to the magnetic beads. The poly(A) RNA binds to the oligo(dT) on the beads. The beads were collected on the side of the tube and washed several times to remove any non-binding material. The washed beads were directly used in the reverse transcription reaction, and a aliquot of the reaction was used for PCR (Rosok et al 1996. Biotechniques 21:114-121)

Compared to the traditional approach using total RNA, this method has offered several advantages:

1. Easy and fast. Bypasses the laborious and capricious total RNA isolation. All the procedures, from poly(A) RNA isolation, reverse transcription to PCR, can be performed on a single day. Many samples can be processed at the same time.

2. Dramatically increases the reliability of DD in loblolly pine. Because the steps in RNA isolation and handling were kept to a minimum and significantly less than the 'total RNA' approach. This allowed us to generate repeatable and clear band patterns on the differential display gels.

3. Very small numbers of embryos are required. This new procedure eliminated the RNA precipitation step, and thus avoids loss of RNA. This is important when the amount of embryos available for RNA isolation is small. Using the beads-oligo(dT) approach, the poly(A) RNA is captured by the oligo(dT) on the beads, no matter how small the amount is. So far, we are able to generate clear DD band pattern of a single stage 7 embryo. This

pattern is almost identical to those of 2, 5 and 10 embryos at the same stage.

Many sets of DD have been performed which generated clear and informative band pattern. We have performed solid-phase RNA differential display on embryos at suspension stage (stages 1 and 2), stage 3, 4, 5, 6, 7 and 9. Eight sets of primer combinations were used for each stage. In a initial step, more than 70 bands on the DD gels were identified that either increase or decrease their abundance from early to late stages. Thirty two of these bands were cut out the DD gels and successfully re-amplified. We have cloned 20 of these amplified sequences into a plasmid vector and screened 200 clones using PCR. More than 100 clones contained a plasmid that carries insert sequence of the expected size. Characterization of the PCR products of these clones using restriction enzymes have identified more than 40 unique sequences. We are in the process of re-examining their abundance at different stage of somatic embryo development, and sequencing.

Future Goals:

1. Confirm/Demonstrate differential expression
2. Continue cloning, characterizing and sequencing more DD bands.
3. Detailed studies of their expression during somatic embryo development.
4. Comparison of their expression in somatic and zygotic embryos.
5. Study gene function and expression regulation.

Preliminary Comparison of Zygotic and Somatic Embryo Development Using Differential Display

Nanfei Xu, Jerry Pullman and John Cairney

The application of differential display to zygotic embryos began recently, a reflection of our confidence that this valuable material could now be analyzed with minimum loss. Results are preliminary but this work, and the comparison of results to somatic embryos, is being vigorously pursued and will be discussed in greater detail in oral presentation.

Previous Achievement:

1. Detailed staging and morphological description of embryo development. This was accomplished by a team of workers over the summer of 1996.
2. Collected embryos of all 18 stages

New Developments:

1. DD has been successfully carried out using as little as 1 mid- to late-stage embryo using the oligo-dT-bead primed method
2. DD performed for embryos of 18 stages, 216 band patterns obtained, many stage-specific bands have been identified.
3. The DD patterns of zygotic embryos were compared to those of somatic embryos. Differences and similarities have been identified. These are currently being analyzed in more detail and will be discussed during PAC presentations.

Future Goals:

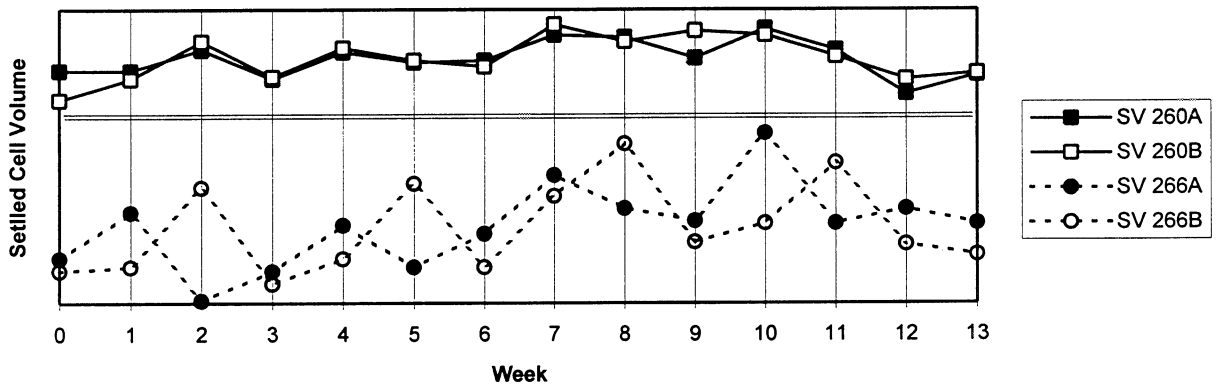
1. Clone and characterize the stage specific bands. We will confirm differential expression and analyze bands by similar methods to those employed in analyzing somatic embryo cDNAs.
2. Long Primers, likely to hybridize to homologous RNA/cDNA from many genotypes can be designed, based on the sequence of the cDNA. These can be used to follow the expression of specific genes in somatic and zygotic embryos of several genotypes.
3. Expression profiles can be established by methods such as RNase Protection.

Examining Cycling in Liquid Culture Somatic Embryos Using Differential Display

Byron Waldrop, Jerry Pullman and John Cairney

During the life cycle of early stage, liquid suspension somatic embryo cultures, certain fluctuations in culture growth can be detected during the weekly sub-culturing routine. These fluctuations are typically measured by settled cell volume, embryo stage and appearance, and cotyledonary yield upon plating. We wished to determine the reasons for these growth fluctuations and have monitored pH, medium osmolarity and gene expression (as reflected by Differential Display banding patterns) over the course of thirteen weeks.

Four liquid suspension cultures representing two genotypes were observed. The plot of settled cell volume shows the cycling fluctuations as peaks and valleys. Genotype 266 showed the most pronounced cycling typically with three weeks between peaks or valleys. Preliminary experiments revealed differences in gene expression during periods of cycling, and these results have been repeated to give a more extensive and reproducible analysis.



We have isolated RNA from tissue samples representing several peaks and troughs and wish to generate a banding pattern for each. Recently we have employed the oligodT bead-primed method of isolating RNA which will allow us to quickly assess gene expression in all genotypes at different points of the cycle. Prominent bands will be isolated, analyzed and cloned. By sequencing these cDNAs we hope to gain insight into the physiological processes which are occurring during culture cycling.

Gene expression during Loblolly Pine embryogenesis: drought-inducible genes also expressed in pine cell suspension culture.

Ranjan Perera and John Cairney

Institute of Paper Science and Technology Forest Biology Group, 500 10th Street Atlanta GA 30318

The Molecular biology research component of the somatic embryogenesis program in our laboratory focuses on understanding gene expression during the course of development in somatic and zygotic embryos. cDNA isolation and identification is reported elsewhere in this report. While those experiments will reveal what is switched on and when, we will subsequently extend that analysis to ask how are these genes switched on and where. In this section, some of the approaches which can be used in genes analysis are explored using partially characterized cDNA clones in our possession.

Some of the earliest molecular biology research on embryogenesis revealed a class of proteins, Late Embryogenesis Abundant (Lea) proteins whose quantities were elevated late in embryo maturation. This stage corresponds with a period when the embryo often becomes desiccated. Prior work revealed that many of these lea proteins are induced in vegetative tissue of mature plants subjected to water stress.

In work initiated in the Labo Voor Genetica in Gent Belgium, Dr. Perera succeeded in isolating a number of cDNA for drought-inducible genes in rice cell culture. Three clones, pOS 1.3, pOS C2, and pOS 1.6, appear useful tools to study gene expression in Loblolly pine.

Though the comparison of each rice sequence with current data-banks did not, initially, reveal any homology for clones for pOS 1.3 and pOS C2, the clone pOS 1.6 shows a 56% homology to a already known abscisic acid inducible glycine rich protein (AAIGP: Gomez et al Science 1988). This gene encodes a protein built up of two domains. The amino terminal domain contains a RNA binding consensus sequence whereas the other is very glycine rich. The later domain has two main motives (GGYGG and RRE) of GGYGG is also present in our sequence. However our rice sequence is still too small (380 bp of cDNA) to confirm the presence of the RNA binding box.

Recent GenBank comparison has shown that clone pOS C2 shows similarity to Lipid Transfer Proteins from a number of plant species. While induced by water stress it is also preferentially expressed in rice cell suspension cultures (Perera unpublished). We would like to apply this knowledge to Loblolly Pine and by identifying studying the comparable pine gene, to determine the features of this gene which are responsible for restricting its expression to a certain stage of development, whether there are hormonal signals involved in this expression pattern and whether different conditions of cultivation can promote the expression of this gene in different stages of embryogenesis. Such a gene would serve as a model for regulation and, along with others isolated from other parts of

the project, would enhance our understanding of how the program of gene expression is regulated during embryogenesis.

We may use the pOS C2 cDNA to establish a number of techniques in the laboratory which will subsequently be employed by others working on the Somatic Embryogenesis project. pOS C2 will be used for

a) Gene expression studies in different stages of embryogenesis
b) Gene expression studies in Pine cell culture where alteration in expression of pOS C2 due to various changes of nutritional, aerobic and anaerobic, temperature, etc.will be monitored.

We have already isolated RNA and DNA from different tree varieties (Loblolly Pine cell suspension culture # 260, cotton wood and sweet gum). This RNA and DNA were electrophoresed in 1% agarose and subsequently blotted onto the nylon membrane. These membranes hybridized with the random-primed probes prepared from cDNA inserts pOS 1.6, pOS 1.3 and pOS C2. Southern and Northern analysis data confirms that these genes are present in Loblolly Pine. The expression of these genes will be followed using techniques such as Rnase Protection assay thus this aspect of the project will both provide new insights into gene regulation and, by establishing gene analysis techniques, furnish the group with tools to further their own work.

HARDWOODS

MASS CLONAL PROPAGATION OF GENETICALLY IMPROVED AND ENGINEERED HARDWOODS

Status Report for
Project F011

Gerald Pullman
John Cairney
Gary Peter
Luis Destefano
Joseph Perera
Barbara Johns
Yolanda Powell
Ken Camp
Tony Wang
Karen Floyd
Cielo Castillo
Alan Harris

March 25-26, 1997

Institute of Paper Science and Technology
Atlanta, Georgia 30318

MASS CLONAL PROPAGATION OF GENETICALLY IMPROVED AND ENGINEERED HARDWOODS

TECHNICAL PROGRAM REVIEW
February, 1996 - February, 1997

Project Title:	MASS CLONAL PROPAGATION OF GENETICALLY IMPROVED AND ENGINEERED HARDWOODS
Project Code:	HRDWD
Project Number:	F-011
PAC:	Forest Biology
Division:	Chemical and Biological Sciences
Project Staff:	**Faculty**: Gerald Pullman, John Cairney, Gary Peter, open; **Postdocs**: Luis Destefano, Joseph Perera
Staff:	Barbara Johns, Yolanda Powell, Ken Camp, Tony Wang, Karen Floyd, C. Castillo, A. Harris,
FY 96-97 Budget:	$166,548
Supporting Research	
M. S. Students:	2
Ph.D. Students:	0
Govt. Grants:	$165,500

LONG RANGE RESEARCH AREA:

Virgin Fiber Supply

PROGRAM OBJECTIVE:

To develop reliable and efficient methods for genetic engineering of hardwoods.

SUMMARY OF RESULTS:

Forest Biology Faculty brought in approximately $ 161,500 in outside funding during the past year for research related to F-011.

Populus deltoides (Clone C175) has been successfully transformed and plants regenerated. The transfer of foreign genes (kanamycin resistance gene, and Gus reporter gene) have been confirmed by positive GUS staining and genomic DNA gel blot analysis.

Established Zinnia xylem formation model for elucidation of regulatory mechanisms controlling secondary wall formation and pattern.

Plant genes for a novel regulatory system (PAM/PGL) have been tentatively identified and cloned.

We have constructed a number of truncated and mutated gene fragments which we will use to assay the basis of RNA stability and stability of a drought-inducible gene.

Flower formation genes for loblolly pine (homeotic genes) have been tentatively identified.

Three year hardwood plan has been proposed which will focus on improved hardwood transformation methods, gene regulation for function of transferred genes, and added tree value by improving fiber quality.

AGROBACTERIUM TUMEFACIENS-MEDIATED TRANSFORMATION OF *POPULUS DELTOIDES* LEAF SECTIONS

Ronald J. Dinus, Camille J. Stephens, Shujun Chang, Karen M. Floyd and John Cairney [1]

[1]Institute of Paper Science and Technology
Forest Biology Group, 500 10th Street NW
Atlanta GA 30318

Abstract

A system was developed for the transformation of a eastern cottonwood clone (*Populus deltoides*). Leaf explants were infected with *Agrobacterium tumefaciens* strain LBA4404 with the binary vector PBI121. Transgenic plantlets were confirmed by their kanamycin resistance, ß-glucuronidase (GUS) activity, and the presence of the *uidA* gene, as demonstrated by polymerase chain reaction (PCR), and genomic DNA blots.

Several factors affected the efficiency of transformation. Long exposure time (120 min), of explant to Agrobacterium tumefaciens, was beneficial. Delayed selection by 14 days increased the transformation efficiency especially when the exposure time was short (30 min).

Introduction

Populus deltoides Bartr. ex Marsh (eastern cottonwood) is one of the most important hardwoods in the world. It is the fastest-growing commercial tree species in North American (Cooper and Van Haverbeke 190x). The rapid growth rate and good pulping properties would make it the ideal fiber source for paper industry, but intolerance to competition and susceptibility to at least 22 different insects and diseases have limited the potential value of eastern cottonwood (Cooper and Van Haverbeke 1990). Genetic improvement has been achieved through conventional selection and breeding. However, the progress has been hindered by the long generation time and the loss of biomass production associated with the selection for disease resistance (Cheliak and Rogers 1990; Ostry and Berguson 1993). Genetic transformation will be the way to overcome the limitations of traditional tree improvement program.

Foreign genes have been successfully introduced into economically important tree species including several poplar species (Fillatti et al. 1987; De Block 1990; McCown et al. 1991; Brasileiro et al. 1991, 1992; Tsai et al. 1994; Confalonieri et al. 1994). Agronomically important genes such as herbicide resistant genes, disease resistant genes, insect resistant genes and genes regulating lignin biosynthesis have been transferred into a few poplar hybrids and quaking aspen (Fillati et al. 1987; Riemenschneider et al. 1988; Klopfenstein et al. 1991; McCown et al. 1991; Brasileiro et al. 1992; Tsai et al. 1994). All of these studies have demonstrated the application of genetic engineering in tree

improvement. In return, transgenic trees showed increase in tolerance to insects and disease, effectiveness in competition control, and quality of fiber. Much of this research, however, was performed with taxa other than *P. deltoides* or its hybrids (e.g. Fillatti et al. 1987). Transformation has also been accomplished with hybrids between *P. deltoides* and other species; e.g, *P. trichocarpa* (Parsons et al. 1986, De Block 1990, Wang et al. 1994) and *P. nigra* (Charest et al. 1992, Devantier et al. 1993). Against this background, we sought to devise a transformation protocol for *P. deltoides*, with the intent of extending it from clones noted for ease of manipulation in culture to elite clones of commercial value.

In this report we describe a system for transferring the ß-glucuronidase gene (*uidA*) and the neomycin phosphotransferase II gene (*npt*II) into an eastern cottonwood clone using *A. tumefaciens* strain LBA4404. The important factors affecting the transformation and regeneration will be discussed.

Materials and methods

Plant Materials, Tissue Culture, and Regeneration Eastern cottonwood clone c175, which was provided by Dr. S. G. Ernst (University of Nebraska-Lincoln), was used in this study. Shoot stock cultures of this clone were propagated by culturing nodal sections to stimulate axillary buds, and maintained *in vitro* on hormone-free WPM medium (Lloyd and McCown 1981) in sterile GA7 boxes. The leaf section-based regeneration system was developed by Uddin et al. (1990). Shoot regeneration were obtained by culturing leaf sections on DKW medium (McGranahan et al. 1987) containing 1µM BA (benzyladenine) and 1 µM NAA (naphthaleneacetic acid). Plantlets were grown at 22-24 ° C in 16 h light/8 h dark photoperiod.

Kanamycin Sensitivity Aseptic c175 leaf sections were cultured on shoot regeneration medium containing a range of concentrations of kanamycin in an earlier study (Shorter 1991). Kanamycin at 30 mg/L stopped the shoot regeneration.

Marker Gene Vector and Agrobacterium Strain A Ti-plasmid based vector PBI121 (Clontech, CA) which contains both reporter gene ß-glucuronidase (GUS) and kanamycin resistant neomycin phosphotransferase (NPTII) gene was used for eastern cottonwood transformation. The *uidA* ('GUS') gene was driven by the constitutive Cauliflower Mosaic Virus 35S promoter (Jefferson 1987), and NPTII gene was fussed with nopline synthase (NOS) promoter (Hoekema et al. 1983). *A. tumefaciens* strain LBA4404 is a disarmed derivative of Ach5 strain (Hoekema et al. 1983). PBI121 was introduced into LBA4404 by the freezing transformation method (Zahm et al. 1984).

Plant Transformation and Regeneration *A. tumefaciens* harboring PBI121 was grown overnight in LB medium with 50 mg/L kanamycin on a rotary shaker (120 rpm) at 25 ° C. The bacterial suspension was centrifuged (2500 rpm, 15 min) and resuspended with DKW liquid media to the desired concentrations. Leaf blades were removed from aseptically propagated stock plants and cut into sections about 0.5 cm^2. Leaf sections

were stocked into petri plates with liquid medium and separated into different treatments. Carbenicillin at 500 mg/L was added to the medium for bacterial control. Kanamycin was used at 50 mg/L for selection. Transgenic shoots were excised and transferred to hormone-free WPM supplemented with 50 mg/L kanamycin for rooting.

Factors Studied Two studies were conducted to identify the important factors affecting transformation. First experiment was to determine if bacterial infection would affect regeneration. In this experiment, 30 leaf sections were incubated with liquid regeneration medium for 5 min and 110 were incubated with bacterial suspension in liquid regeneration medium at the concentration of 10^9 (cells/ml) for 5 min. Leaf sections were block dried before they were transferred to solid regeneration medium with 500 mg/L Carbenicillin. Second experiment was in a factorial design to see the effects of bacterial concentration, time of incubation, and time of selection on transformation efficiency. Bacterial concentrations were 10^7 and 10^{10} (cells/ml). The incubation time varied from 30 min to 120 min. Selection was compared from immediate selection verses delayed section for 14 days (named as Delayed Selection). Each combination contained 30 leaf sections.

Enzymatic Assay for GUS Activity Callus and leaf tissues were assayed for GUS activity *in situ* with 5-bromo-4-chloro-3-indoyl glucuronide (X-GLUC) as described by Jefferson et al. (1987). Putative transformants were considered when callus is actively growing on selection medium and stained positive for GUS.

DNA Extraction, PCR Analysis and Genomic Blot Analysis DNA isolation and PCR assay as following: individual calli or leaves were put into microcentrifuge tubes and flash frozen by liquid nitrogen before they were grounded to powder with a stirring rod, 500 µl of hot (60-65 C) extraction buffer (Chang et al. 1991) was added to the tubes and tubes were incubated for 15 min at the same temperature, the mixtures were then extracted twice with choloroform:isoamylalcohol (24:1), supernatants were mixed with 2 volumes of absolute ethanol and stored at ≤ 20 C to precipitate DNA, after centrifugation to pellet the DNA, DNA pellets were washed twice with 75% ethanol, vacuum dried and dissolved into water before the quality and quantity were checked by electrophoresis. Approximately 100 ng of DNA was used as templates for PCR amplification of a 1.25 kb GUS fragment. The sequence for the forward primer was: GGT ATC AGC GCG AAG TCT TT; and for the reverse primer was TGC GTG ATG ATA ATC GGC TG. PCR reaction buffer contains 50 mM KCl, 10 mM Tris-HCl (PH 9.0), 1% Triton X-100, 0.25mM dNTPs, 1 mM MgCl2, 50 pmoles of each primer, and 1 unit of Taq DNA polymerase (Promega, WI). The PCR conditions were; 250ng DNA. Tthe cycle conditions were: 95°C, 5 min; (95 °C, 1 min; 45 °C, 1 min; 72 °C, 1 min) 35 cycles; 72 °C 5 min.

High molecular weight DNA was extracted from control plants and transformants for genomic blot analysis by the method of Doyle and Doyle (1990). In three separate reactions 10 micrograms of genomic DNA was digested with BamHI, EcoRI and HindIII, enzymes which cut T-DNA one time. Digested DNA was separated by electrophoresis

and transferred to a nylon membrane (Sambrook et al 1989). The coding region of the uidA gene was used a probe and hybridization was detected using the Gene Images non-radioactive detection system (Amersham, La Jolla, CA)

Results and discussion

Agrobacterial Infection Delays the Course of Regeneration The first experiment was designed to investigate the effects of *Agrobacterium* infection on callus, shoot primordia, and shoot formation. Similar to our observation in earlier trials (data not shown), *Agrobacterium* infection delayed the development of callus, shoot primordia, and shoots (Fig. 1a and 1b). The biggest delay happened at the stage of callus formation. It took about 2 weeks longer to have the same percentage of explants develop callus (Fig. 1). Similarly, shoot primordia and shoot formation were also delayed. The delay in callus formation indicated that the number of cells responding to treatment was reduced and the cells were more vulnerable after the bacterial infection. Since the delay in callus proliferation was about two weeks, we tested whether a Delayed Selection process, a delay in Kanamycin selection for 2 weeks, would increase survival of transgenic cells

Factors Affecting the Transformation of Eastern Cottonwood Three factors, the concentration of *Agrobacterium*, time of incubation, and the way to apply kanamycin selection, were considered in the transformation experiment. Nearly 50% of explants developed GUS positive and kanamycin resistant calli (Table 1). Fifteen percent of explants had GUS positive shoots regenerated from the kanamycin resistant calli (Table 1). Overall there were more kanamycin resistant calli (average 37.5% of explants) and GUS positive shoots (average 9%) from Delayed Selection treatment than direct selection treatment (27.5% and 5% respectively). Delayed Selection increased the transformation efficiency significantly when the bacterial infection time was 30 min, but did not help the transformation when the infection time was 120 min (Table 1). Longer exposure time (120 min) was more effective than shorter exposure time (30 min) in transformation when the selection was direct (Table 1). However, it did not improve the transformation when selection was delayed, though it increased the average percentage of putative transformants by 50% (45% verses 30%). The bacterial concentration did not affect the transformation efficiency.

Table 1. Effects of bacterial concentration, incubation time and selection method on transformation efficiency. Data collected 91 days after the transformation.

Selection %	Exposure Time (min)	Bacterial (cells/ml)	No. Explants	% Explants Having GUS$^+$, Kan^R	Avg.% Calli	%Expalnts with GUS$^+$, Kan^R	Avg. Shoots
Direct	30	10^7	30	10	*23.5*	0	
		10^{10}	30	37		0	*0*
	120	10^7	30	36		10	
		10^{10}	30	27	*31.5*	10	*10*
				Average %	**27.5**		**5**
14-day Delay	30	10^7	30	27	*30*	10	
		10^{10}	30	33		10	*10*
	120	10^7	30	43		3	
		10^{10}	30	47	*45*	13	*8*
				Average %	**37.5**		**9**

After the kanamycin resistant and GUS positive shoots were elongated. The presence of the *uidA* gene was checked by isolating DNA from transgenic plants and conducting PCR. A 1.2kb fragment was generated, the predicted size for correct amplification (Fig. 2). The presence of integrated copies of the uidA gene was confirmed by Southern hybridization. Four hybridizing bands are shown in Figure 3, these most likely represent four separate integration events.

In this system, GUS positive callus was not reliable, but GUS positive shoots were very reliable because of the confirmations with PCR and Southern analysis. This is not surprising because some calli would fail to develop into shoots even in the controlled regeneration system. After three months on selection medium, the untransformed calli were mostly very sick and dying. Only the calli adjacent to transgenic calli would still show kanamycin resistant growth, but they never developed into shoots (data not shown). In this study, almost all the transgenic shoots were from transgenic calli, though some transgenic calli did not develop into shoots. The rate for escapes was very low.

Low rate of escapes seemed to be related to the regeneration system and the kanamycin selection. In our regeneration system, low levels of BA and NAA were used at the same amount (1μ M each). The amount of growth regulators were considerably lower than some other poplar regeneration systems (Brasileiro et al. 1992; Confalonieri et al. 1995). There are two characteristics for this regeneration system: (1) Regeneration is not as vigorous as when higher amount of growth regulators (e.g. 5 μ M) are used (data not shown); (2) Callus and shoots develop at the same time on the same medium. Consequently, the regeneration is more sensitive to selection, and does not allow callus to grow vigorous either. In addition, 50 mg/l of kanamycin is nearly twice the lethal dosage

for untransformed leaf sections. All these factors could have contributed to the low level of escapes.

Of three factors tested, Delayed Selection and time of incubation are important for transformation, whereas bacterial concentration does not have significant effects on transformation efficiency. Delayed Selection allowed more transgenic cells to survive, thus resulted a higher transformation rate. The effects were obvious at shorter bacterial incubation time, but not clear when the bacterial incubation time was long. Longer incubation could have given the bacteria more chance to transfer the DNA to more plant cells, but it only helped when the selection is direct. It is not clear why their is not any synergistic effects from Delay Selection and longer bacterial incubation. More data needs to be accumulated to answer this question. As in the transformation of black poplar, bacterial concentration was not an important factor in transformation of c175. Thus, lower concentrations have been used for later transformation works for convenience.

Conclusion

This study reports the successful *Agrobacterium*-mediated transformation of *P. deltoides* Bartr. ex Marsh. Stable transformation was obtained as high as thirteen percent of the explants cultured. Best results were obtained by either 120 min bacterial incubation followed by direct selection or by 30 min bacterial incubation with selection delayed for 14 days (Delayed Selection). We have used Delayed Selection and 30 min incubation with bacteria at around 10^8, and consistently obtained transgenic plants. This system will give the chance to engineer the eastern cottonwood clone for improved value such as stress resistance and wood quality.

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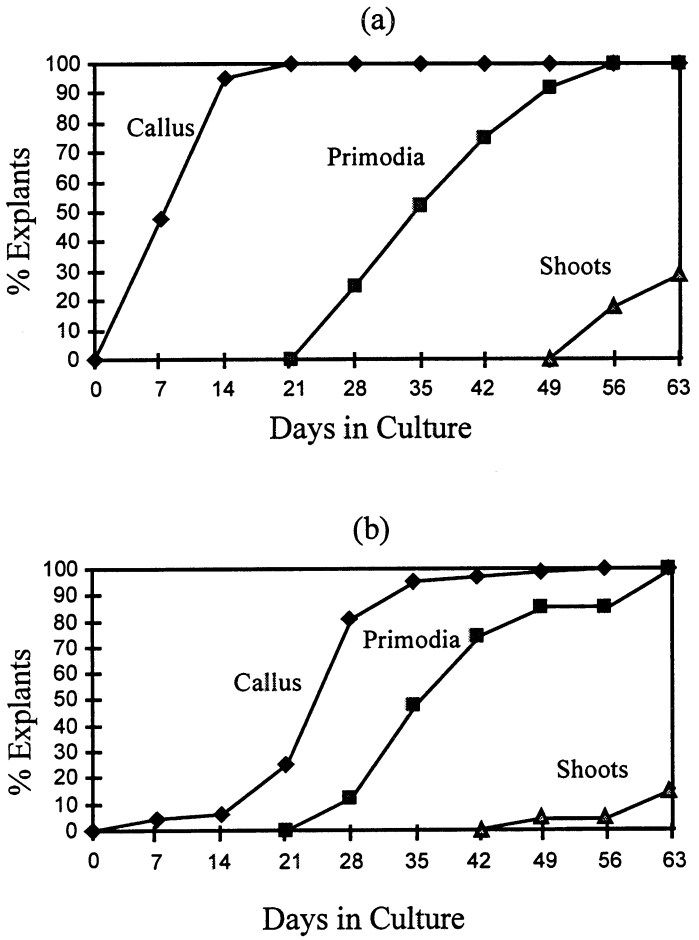


Fig. 1. Percentage of leaf explants had callus proliferation, shoot primordia development and shoot regeneration on regeneration medium. (a): normal development; (b): infected by 10^9 cells/ml *Agrobacterium* but not selected by kanamycin.

Figure2: GUS Staining of Transformed Cottonwood

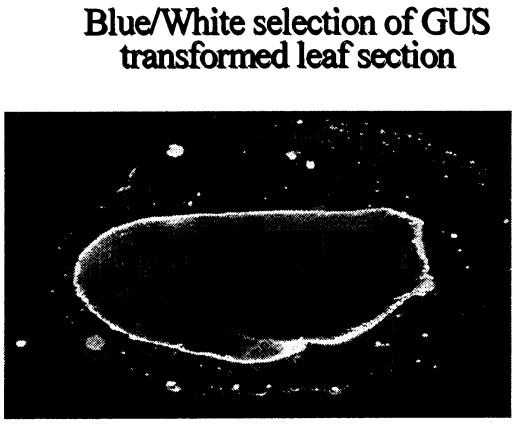


Figure3 : PCR of GUS gene from putatively transformed Cottonwood. Fragment of predicted size is indicated by arrow.

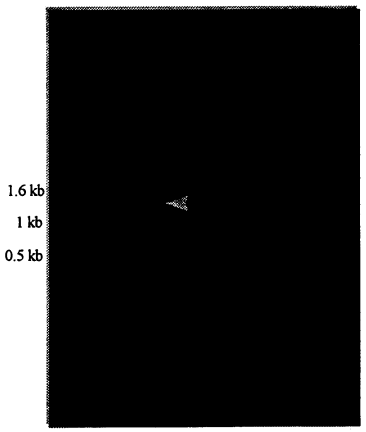
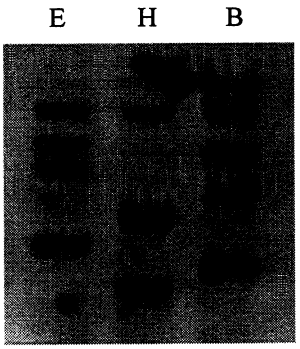


Figure 4. Southern blot of DNA from transformed Cottonwood using *uidA* gene as probe

Southern Blot of Transgenic Cottonwood DNA. DNA was cut with EcoRI, HindIII and BamHI and probed with fragment of uid gene. Hybridzing bands ranging from 6 kb to 15kb.



Regulation of Expression of a Stress-related Gene: Promoter and 3' UTR Analysis

Luis Destefano and John Cairney

The efficient and stable expression of a gene in a transgenic plant is dependent of the presence of several molecular determinants including a transcriptionally-efficient 5'-regulatory region, a 5' untranslated leader sequence, a translational start sequence (AUG) with a plant favorable context, and a transcription termination/polyadenylation sequence. These and other determinants (i.e. codon usage) contribute to mRNA and protein production at the right tissue and/or state of development of the plant. However, a fine tuning of transgene expression has to evaluate other parameters such as stability and turnover of both mRNAs and their translated products. Overall these components are important to consider in a strategy to develop a plant vector.

A proteinase inhibitor (PI) gene which is induced under drought stress is being studied and its promoter and 3' untranslated regions analyzed in detail. Previous work included the characterization of several cDNA forms of this gene as well as two genomic clones.

Characterization of Two PI Promoters. One of the most frequently used methods for characterizing plant regulatory elements is deletion analysis. By this method the DNA fragment containing the regulatory element is treated with an exonuclease to generate a serial deletion of the promoter region. Alternatively, the deletion mutants can be obtained by removal of a restriction fragment from the regulatory region. The deletion mutants are then spliced upstream of a reporter gene whose expression can be easily monitored by either transient assays or stable transformation.

Two genomic clones, 18-1 and 12-95, contain promoter sequences of ca. 1.3 and 2.4 kb respectively. Several constructs including full-length and deletion mutants of these promoters have been obtained and fused to the GUS reporter gene. In both cases the GUS gene was fused in-frame a few codons downstream from the AUG initiator codon. Similarly, two extra constructs contain the full length promoters, the 5' untranslated region, the first exon, the first intron and a few codons of the second exon fused in-frame upstream of the reporter gene.

The promoter of the 18-1 gene contains several single restriction sites that have resulted in the production of the following deletion mutants expressed as the distance in base pairs from the transcription initiation: -1397 (full length), -949, -794, -558, -254, and -146. Additionally, there is an A-rich region (60/108), including a row of 30 A's, between -254 and -146 that has been deleted from a full length construct in order to evaluate its role as a putative novel regulatory element. Similarly, deletion mutants for the 12-95 promoter gene include: -2400 (full length), -1800, -990, -541, -266, -133, and -101. The borders of all constructs have been confirmed by sequencing.

All constructs based in the pBIN19 binary vector will be transferred to plants via Agrobacterium. Expression of the reporter GUS gene will evaluated in planta under normal and stressed conditions. Selected cassettes containing /Promoter fragment/GUS/NOS 3'/ based in pUC derivative vectors will be used in transient assays using Arabidopsis and Atriplex cell suspensions.

Characterization of a 3' UTR element. Previous work in our lab showed that the PI gene family could be grouped into two classes according to the presence or absence of an AU-rich region in their 3'UTR end. One of the genomic clones, 18-1, contains such a motif in its 3'UTR region. A similar approach used for the analysis of the promoters has been used to analyze the role of such region in mRNA stability and/or translatability. Several 3' to 5' deletions of the region after the termination codon have been placed downstream of the cassette CaMV35S/GUS using single restriction sites. Standard cloning techniques have resulted in the production of the following deletion mutants expressed as the distance in base pairs after the termination codon: + 187, +361, +583, +766, +1005 and +1574. As before, the pBIN19-based constructs will be introduced in planta via Agrobacterium and selected pUC-based constructs will be used in transient assays in either Arabidopsis or Atriplex cell suspension cultures. Different effectors will be tested to monitor any effect in the chimeric GUS mRNA stability and/or translatability: ABA, PEG, MeJ, temperature and salt stress.

Establishment of Arabidopsis and Atriplex cell suspension cultures. We will evaluate these regulatory regions in transgenic plants, however, rapid and informative assays may be accomplished through transient assay. For this reason, cell suspension cultures were established for Atriplex, the plant from which these genes were derived, and Arabidopsis, as a model plant. Arabidopsis and Atriplex seeds were germinated in vitro and different seedling explants were transferred to callus induction media (Gamborg B5 and WNA plus hormones respectively). After several weeks friable calli were transferred to liquid media. Successive transfers are being made to obtain finer cell aggregates.

Identification of A Plant Enzyme Which Activates A Regulatory Peptide

John Cairney[1], Jerry Pullman[1], Karen Floyd[1], Dana Freeman[1], Luis Destefano[1], Sheldon May[2], Charlie Oldham[2], Allison Moore[2]

[1]Institute of Paper Science and Technology, Forest Biology Group, 500 10th Street NW Atlanta GA 30318
Department of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta,

An new collaborative project with Georgia Tech has been initiated as an A190 Project The project seeks to find a Plant homologue of a regulatory enzyme which enzyme regulates the activity of many neuropeptides in mammals. We have preliminary evidence that a similar enzyme exists in Pine and Arabidopsis

The PAM-PGL Enzyme System

Many regulatory peptides require modification (amidation) at their carboxy terminal in order to become physiologically active. **PAM** (Peptidylglycine a-amidating Monooxygenase) and **PGL** (Peptidoaminoglycolate Lyase) work in concert to modify and activate certain enzymes. Such Mammalian enzymes include calcitonin, growth hormone releasing factor, LH-RH (luteinizing hormone releasing hormone), vasopressin, gastrin, a-MSH (a-melanotropin). The production of C-terminally amidated peptides can be better achieved by a two-step process in which, in the first step, a non-amidated peptide is produced which is, in the second step, amidated at its C-terminus.

The monooxygenase, PAM, forms the a-hydroxyglycine derivative of the substrate peptide, and the PGL catalyzes the dealkylation step to form the amide product and glyoxylate (Fig. 1)

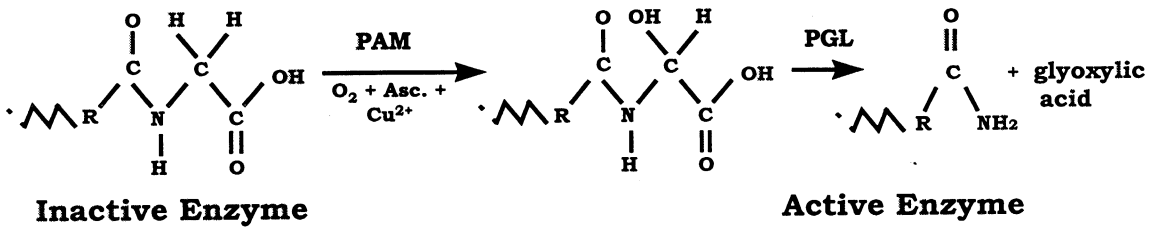


Figure 1. Mechanism of PAM-PGL system.

The PAM and PGL enzymes are produced as a single peptide which is then cleaved into the two activities. Several cDNA and genomic clones of PAM-PGL have been isolated from mammalian sources and gene regulation has been investigated.

This projects is jointly undertaken with GIT and provides an opportunity to determine novel forms of regulation, to establish active collaboration with GIT and to win external support.

Early cell culture work showed that an inhibitor of PAM-PGL inhibits growth of pine somatic embryos. We pursed this work and conducted a number of Southern blots to establish the presence of a PAM-PGL homolog in plants. We generated a 500bp fragment of PAM from by PCR from a baculovirus clone from Dr. Mays lab and probed DNA from Loblolly Pine and Arabidopsis. The Loblolly Pine gave a smeared signal, the Arabidopsis gave more specific bands.

This seemed encouraging evidence that a similar gene was present in plants and we decided to screen a library. We had no idea as to when and where the gene was expressed so cDNA libraries were ruled out a source for the clone. We have attempted several RT-PCR experiments using RNA isolated from Loblolly Pine Cell Suspension culture, which we thought may be a likely source of action for a regulatory peptide but to date have had no success.

We have recently focused our attention on obtaining a genomic clone of the 'PAM-PGL' homolog from Arabidopsis. We did so because,

- Arabidopsis has a small genome with little repetitive DNA; genes are relatively easy to find
- Genomic libraries are available free of charge from the Arabidopsis Stock Center
- Genomic clones obtained from Arabidopsis will be a useful probe for isolating other plant copies e.g. from Loblolly Pine

Our initial screens yielded several faint signals and the clones which gave these were isolated for re-screening. Stronger signals would make library screening easier and more certain. Towards this end we have now obtained a full-length bovine cDNA clone from Dr. Richard Means, Johns Hopkins University. We are currently rescreening the library and about to subject the clones we picked earlier to a second round of screening.

Three Year Research Plan for Mass Clonal Propagation of Genetically Improved and Engineered Hardwoods 1997-2000

Gary Peter, John Cairney, Gerald Pullman

Executive Summary: This plan represents our effort to develop an integrated program that addresses research areas of high value to member companies both now and in the future. The proposal attempts to balance the unique strengths of IPST faculty as a whole with our specialized expertise in plant tissue culture, gene regulation, gene identification and xylem tracheary element differentiation to achieve important goals defined both by the research lines of IPST and by member companies. The plan proposes 80% of the work on cottonwood and 20% on other species as models. This overly ambitious plan was written to focus discussions on which are the most important directions and goals for the hardwood program at IPST.

Objectives:

1. To increase the efficiency of transformation methods used for commercially important cottonwood genotypes from the west and the southeast
2. To identify and characterize robust gene regulatory sequences that can be used for stable gene expression in forest trees independent of transformation event, tissue type or cell type
3. To isolate and characterize gene regulatory sequences that direct expression in cambial meristem cells and differentiating xylem cell types leading toward the regulation and control of valuable fiber cell properties
4. To isolate genes that regulate valuable fiber properties e.g., secondary cell wall thickness, cellulose microfibril angle

Recent History & Accomplishments: The hardwood program focused on developing methods to transform cottonwood. IPST researchers lead by Ron Dinus were one of the first to transform *Populus deltoides* or Eastern cottonwood. An organogenic regeneration method using stem tissue was developed for a model clone, C175, which originated in Minnesota and was obtained from the University of Nebraska. This genotype was transformed with the GUS reporter gene using *Agrobacterium tumefacians*. Transformed plants resistant to kanamycin were regenerated. The introduction of foreign DNA was confirmed both by positive GUS staining and genomic DNA gel blot analysis.

Work towards understanding gene regulation of both hardwoods and softwoods is being led by John Cairney. A number of drought induced genes were isolated from the desert shrub *Atriplex canescens*. The structure and function of these genes and the proteins they encode are being characterized. Work towards producing sterile and early flowering tree species has begun with funds from the Georgia Consortium for Technological Competitiveness in Pulp and Paper. The approach being followed is to isolate floral meristem and organ regulatory genes and to inhibit their expression with antisense or dominant negative protein strategies. The focus of our research is to isolate floral meristem and organ regulatory genes from *Pinus taeda*. Such clones will be a resource which can be explored through collaboration with other groups and will provide the basis for an externally funded project. We intend to leverage these state funds to improve our hardwood transformation efforts.

Future Visions: The forest biology team visualizes a state of the art hardwood program which will use commercial cottonwood lines to focus on 1) improving transformation frequency, 2) understanding gene regulation so researchers can add foreign genes and cause them to function where and when desired, and 3) to target fiber modification as value added changes. It is expected that this program will not only result in improved techniques but also create valuable cottonwood clones with modified growth and fiber properties.

I. Vegetative Propagation

Goal: To develop efficient in vitro *regeneration methods that are useful for genetic transformation of* Populus *species*

Year 1- Initiate *in vitro* regeneration experiments with commercially relevant *Populus* clones from the northwest and southeast

1. Identify and select cottonwood clones
   a) Select one model *Populus* hybrid clone from the northwest
   b) Select 1-2 *Populus deltoides* clones that represent elite genotypes for the southeast
2. Acquire cuttings and seeds, initiate plants for explant material
3. Develop culture conditions that stimulate organogenesis/regeneration in new clones
   a) Test standard methods developed with C175
   b) Adapt method for commercially relevant *P. deltoides* genotypes. Test explant type, media composition, hormone concentrations....
4. Initiate transformation studies with *Populus* hybrid clone from the northwest (see section II for details)

Year 2- Initiate transformation studies with *P. deltoides*

1. Continue to improve regeneration systems for *P. deltoides* clones
2. Regeneration system suitable for transformation target

Year 3- Acquire additional elite cottonwood clones and establish robust regeneration methods for new clones that show promise from breeding studies

1. Establish connections with cottonwood breeding programs
2. Obtain new cottonwood clones
3. Initiate regeneration tests with more valuable cottonwood clones

II. Genetic Transformation of *Populus* Species

Goals: To develop vectors and methods useful for efficient selection of transformed Populus *tissues*

Year 1- Initiate transformation of *Populus* hybrids

1. Begin transformation experiments with *Populus* hybrid testing various drug resistance genes and selection schemes, e.g., neomycin phosphotransferase, phosphinotricin, acetolactate synthase, ...(see section III for details)
2. Initiate cell suspension of cottonwood cell line for transient assay work

Year 2- Initiate transformation of *P. deltoides*

1. Transform *P. deltoides* with *A. tumefacians* and test efficacy of promoter selectable marker combinations in transformation procedure, begin to establish rapid and efficient methods
2. Transformation target for elite clone: 5-10% success

Year 3- Improve transformation procedures with *Populus* clones and if necessary expand range of genotypes in the program

III. Gene Regulation

Goals: To develop techniques for use in cottonwood improvement through genetic engineering. Develop nonproprietary vectors for stable, constitutive, high level expression independent of tissue and transformation event. Isolate sequences useful for targeted expression within the cells of the cambial meristem and xylem cell types.

Year 1- Construct nonproprietary sequences for stable high level expression of transgenes useful for transformation studies and agronomic trait introduction

1. Isolate strong, constitutively expressed gene from *Populus* cells to drive selectable marker gene expression that will not be proprietary like CaMV 35S-neomycin phosphotransferase
    a) Isolate cDNA expressed strongly in proliferating tissues; e.g., ribosomal proteins, translation factors, cell division cycle genes, actin, tubulin, prove expression pattern and level by RNA gel blots and *in situ* hybridization
    b) Isolate gene and define start site of mRNA
2. Construct expression cassette to test promoter expression with green fluorescent protein (GFP) or β-glucuronidase (GUS)
3. Identify sequences that may affect stability and translatability of foreign mRNAs.
    a) Continue investigating the functionality of AU rich sequence identified from drought induced gene in stabilizing foreign mRNAs in transient assays with cottonwood cell lines and model plants
    b) Determine whether viral translational enhancers improve the translatability of foreign mRNA in transient assays in cottonwood cell lines and transgenic cottonwood trees

Year 2- Isolate cell type specific cDNAs from the secondary vascular tissues of cottonwood to ultimately obtain a panel of regulatory sequences that confer cell type specific expression patterns in secondary xylem tissues

1. Construct cDNA libraries to secondary vascular tissues of cottonwood at various points in the growth season
2. Begin isolating cDNAs that are expressed specifically in cambial meristem cells, xylem fibers, xylem tracheary elements, vessel elements, ray cells, parenchyma cells....
3. Begin creating transgenic cottonwood trees that test the functionality of high level expression elements

Year 3- Integrate understanding of transcriptional and post-transcritional gene regulation to create improved plant transformation vectors for optimal tissue specific expression.

1. Evaluate these vectors in transgenic cottonwood and model plants
2. Evaluate transgenic cottonwood for quantitative effect of sequence elements in mRNA stability and protein expression

IV. Isolate Genes that Regulate the Rate of Cell Division in the Cambial Meristem, Xylem Differentiation and Fiber Cell Properties

Goals: Isolate genes that can stimulate the rate of cell divisions in the cambial meristem. Identify genes that regulate xylem tracheary element cell size and secondary cell wall properties such as wall thickness and cellulose microfibril angle.

Year 1- Explore approaches for creating transgenic cottonwood with improved cambial growth and fiber properties and characterize fiber properties of short rotation cottonwood.

1. Investigate the feasibility of using conserved sets of genes that when overexpressed in cottonwood are predicted to stimulate the rate of cambial cell divisions, promote xylem tracheary elements or fiber differentiation, stimulate xylem cell elongation...
   a) targeted genes might include cell division cycle genes, hormone biosynthetic genes, hormone response genes
2. Continue development and use of *Z. elegans* as a model system for isolating and elucidating the mechanisms that regulate tracheary element differentiation and fiber properties
   a) Develop culture methods, transient assays and microscopic analyses to enhance utility of the *Z. elegans* model system
   b) Analyze rac GTPase-proteins investigating their role in regulating cytoskeletal organization and directing cellulose synthesis in *Z. elegans*
   c) Isolate cellulose synthase gene(s) from *Z. elegans* tracheary elements and develop tagged version of cellulose synthase for identifying other subunits, subcellular/membrane localizations, interactions with microtubules.
3. Isolate cellulose synthase gene(s) from cottonwood cambium and differentiating xylem cells
4. Develop links with experts at IPST to measure wood and fiber characteristics of short rotation cottonwood

Year 2- Utilize *Z. elegans* for testing the effect of overproducing cellulose synthase subunits during tracheary element differentiation and begin investigating the mechanism by which microtubules interact with the cellulose synthase complex. Continue with genetic engineering strategy for enhancing growth of the cambial meristem in cottonwood

1. Test if overexpression of the cellulose synthase catalytic subunit in differentiating xylem tracheary elements of *Z. elegans* increases cellulose production
2. Create cottonwood cell line expressing epitope tagged cellulose synthase and immunoprecipitate cellulose synthase complex from purified plasma membranes
   a) Initiate biochemical analyses of cellulose synthase complex
   b) Investigating cellulose synthase interaction with cytoskeletal components
   c) Look for presence of rac GTPases in cellulose synthase complex
3. Isolate cDNAs and their corresponding genes which are expressed in specific xylem cell types
4. Transform cottonwood with gene that will stimulate the xylem production or cell division rates of cambial cells

Year 3- Implement strategies proven with *Z. elegans* to improve fiber properties by creating transgenic cottonwood plants. Analyze the properties of the cambium and secondary xylem in transgenic cottonwood

1. Transform cottonwood gene(s) to enhance cellulose production and/or cellulose microfibril angle
2. Analyze transgenic cottonwood created in year 2

Progress Towards Hardwood Plan

1. *Identifying cottonwood clones for developing methods for regeneration and transformation.* Our plan is to get one northwest hybrid that is known to respond well in tissue culture so that we can establish hardwood transformation of commercially important cottonwoods. In addition we need to identify and begin tissue culture experiments for a couple of important cottonwood clones that have commercial relevance in the southeast. Overall the most important species in the southeast is *Populus deltoides.* Eastern cottonwood is more disease resistant than most of the other species and therefore does better in the southeast than any of the hybrids from the northwest. Numerous discussions with cottonwood breeders in both universities and companies convinced us that no one clone stood out dramatically from all of the rest. This of course is due in large part to the relatively restrictive growth conditions that cottonwood clones do well under. However, another reason suggested was that there hasn't been a sustained, long-term breeding effort in the southeast for cottonwood. The Stoneville clones from the mid 60's and 70's, developed to grow in the Mississippi basin, represent one of the best efforts, but the original orchard declined due to lack of funds and was decimated recently by unusually cold weather. With renewed interest in cottonwood as a species for tree farming, a recent effort has been initiated to collect, establish duplicate orchards and test a large number of natural cottonwood variants. This effort is in its early days and is being led by Dr. Sam Land at the University of Mississippi.

   Two of the most highly recommended *P. deltoides* clones for us to begin with are Stoneville 66 (St66), a proven clone which is the best of the Stoneville selections on volume, specific gravity and cellulose content. The other is S7C1, which has been tested for growth in a wide range of sites throughout the southeast, and is one of the better performing clones from the study being headed up by Sam Land from the University of Mississippi.

   One initial tissue culture test was attempted with Stoneville 66 and reported in the 03/95 PAC report, pg. 111. St66 responded reasonably well to the culture conditions used for C175, with 20% of the explants giving harvestable shoots from adventious buds which could be rooted. This provides an excellent starting point for our proposed transformation efforts with *P. deltoides.*

2. *We have established the* Z. elegans *system here at IPST*: ~50% of the cells differentiate into tracheary elements within 72h. This is similar to the timing and percent differentiation achieved previously at the University of California, Berkeley. Alternate culture conditions are being tested to achieve even higher rates of differentiation and also to increase the efficiency of the transient assay system.

STUDENT RESEARCH - COMPREHENSIVE LIST

Following is a list of students in the Forest Biology Group along with their project or thesis titles and a summary of the work proposed or in progress. Projects with a * are specifically targeted at the Softwoods Project.

Levi Barclay (M. Sc.) * First year student.
Title: Bioactive Characterization of Embryo Development Using Antibody Staining Techniques
Advisor: Gary Peter
Summary: Antibody staining techniques will be used to identify specific proteins that are activated during embryo development. The research will relate morphological development with the biochemical changes that occur during embryo development. The ultimate goal is to identify markers, or regulatory changes, which are predictive of the developmental potential of somatic embryos. These markers and rapid staining techniques will be used to determine the quality of somatic embryos propagated from various genotypes and grown in various culture conditions. It is expected that these antibody markers will accelerate research directed towards improving the quality of somatic embryos.

Karen Crews (M. Sc.) * First year student.
Title: A survey of Anthraquinones in Commercial Trees and their Potential Release from Chips during Pulping
Advisor: Jerry Pullman and Don Dimmel
Summary: Anthraquinones (AQs) can be used at extremely small amounts to improve pulping productivity and environmental factors. AQ increases pulping rates and product yields, removing greater amounts of lignin during pulping. Presently an AQ extract has been found in teakwood as well as several other angiosperms. One goal of this project is to expand our knowledge of AQ content in other tree species. Knowing if commercial tree species contain AQ may give us the knowledge that the pathway for AQ production is present. This will allow us to perhaps "turn up" the particular gene for AQ production in major pulping tree species. Using the trees that contain AQs to facilitate pulping of loblolly pine or another major pulping softwood would be the next step. A tree containing AQ components could be added to a major softwood pulp source, such as loblolly pine. These could be pulped together to promote delignification of the latter. Alternatively, liquors from a hardwood cook that contained AQs could be added to a softwood cook. Such experiments will be done with teak and pine. Other comparisons that will be made will be between known woods that are easily pulped and woods that are hard to pulp. If AQ is determined to have an effect on the ability to pulp commercial trees using the above experiments, altering the gene that produces AQ to generate more would allow for faster and more productive cooks for both AQ containing trees as well as non-AQ containing trees. The discovery of new pulping techniques, as well as a broader knowledge of the content of tree extracts, are possible benefits from this research.

John Ceranski (M. Sc.) First year student.
Title: Fluorescent Microscopy: A Tool for Determining the Spatial Distribution of Secondary Wall Components Remaining during Kraft Pulping of Loblolly Pine
Advisor: Earl Malcolm and Gary Peter
Summary: The goal of this study is to determine the sequence of lignin and hemicelluloses removal from specific cell types, regions of cells and secondary cell wall layers during Kraft pulping of *Pinus taeda.* The removal and spatial organization of the remaining components at various times during pulping will be characterized with fluorescent probes to specific cell wall components and fluorescent microscopy in thin sections of pulped pine.

Cristine Estes (M. Sc.) * Second year student.
Title: Free amino acid levels in developing zygotic embryos of *Pinus taeda*
Advisor: Jerry Pullman
Summary: Plant tissue culture is the link between genetic advances made in the laboratory and improved trees and forest products. The goal in tissue culture is to copy high-value trees through somatic embryogenesis. The procedures involved in tissue culture are both time and labor intensive, so methods of improvement to increase embryo quality and yield are desirable.

One of the critical aspects of somatic embryogenesis development is the media on which embryos are grown. Because somatic embryos have been removed from their natural environment, they rely on media to provide them with all the nutrients, hormones, minerals, etc. required for proper development. The media is influential in two aspects of embryonic growth and development; first, it provides nutrients to feed the embryo, and second, it influences the water potential of the system through the solute potential.

Among the compounds present in the natural system are amino acids, or protein precursors. These amino acids are suspected to play crucial roles in both feeding the embryo by providing building blocks for growth and development and in determining the solute potential of the osmotic system. Identification of which free amino acids are present in the female gametophyte and the embryo itself at different developmental stages could provide information on which specific amino acids are essential at which stages.

In this project, frozen embryos and female gametophytes of two clones of *Pinus taeda* will be analyzed for free amino acid content. Embryos will be collected at precise stages of development, stored at -70 degrees Celsius, and analyzed for free amino acids in the system. Free amino acids are available to the embryo for growth and are able to influence osmotic potential in the seed; however, bound amino acids cannot affect embryos in the same ways and will not be measured. By knowing which free amino acids are present in the natural systems, tissue culture media for laboratory somatic embryos can be formulated with specific amino acids to better mimic the natural osmoticants and precursors for growth and development. If tissue culture procedures can come closer to matching the natural processes of embryo development, improve embryo quality and yield could result.

Dana Freeman (M. Sc.) * Second year student.
Title: The Role of Peptide Processing in Plant Cell Growth and Development.
Advisor: Jerry Pullman and John Cairney
Summary: Bioactive peptides are involved in practically every cellular function in animal cells, but in the cells of plants, it is just now becoming apparent that peptides play a part in cell growth and development. Bioactive peptides are generated from precursors through post-translational modifications, in particular, carboxy-terminal amidation. The amide group is required for bioactivity and is speculated to play a role in receptor binding and regulation. Two "amidating enzymes", Peptidylglycine a-Monooxygenase (PAM), and Peptidylamidoglycolate Lyase (PGL), have been demonstrated to function in carboxy-terminal amidation of glycine-extended substrates. A number of substrates, inhibitors, and assays have been developed to detect the presence of PAM and PGL enzymes.

A recent discovery of a 37 amino acid peptide isolated from soybean was found to stimulate the phosphorylation activity of a soybean receptor protein, suggesting a role for the peptide in signal transduction. The peptide has a glycine at its C-terminus, and via mass spectral evidence it is revealed that a portion of the peptide is processed to delete this terminal glycine. Through this evidence, it is highly suggestive that amidative processing of bioactive peptides may occur in plant cells.

The objective of this research is to explore whether amidative processing of bioactive peptides occurs in plant cells of both angiosperms and gymnosperms. The research will be carried out with Eastern cottonwood (*Populus deltoides*) and Loblolly pine (*Pinus taeda*). Research with cottonwood seems ideal because the first finding of bioactive peptides in plants was reported for soybean, another dicotyledonous Angiosperm. Loblolly pine embryos exhibit several measurable variations (cell elongation, cell wall development, cell differentiation) that may possibly be altered in the presence of amidation inhibitors. The goal of the research is to obtain evidence for bioactive processing and to determine what consequences inhibition of such processing may have on plant cell development and growth. The experimental approach will be to make use of the selective and potent amidation inactivators and inhibitors by determining their effects on plant cells in culture.

Jeff Grass (M. Sc.) * Second year student.
Title: Molecular Characterization of Floral Homeotic Genes Expressed in *Pinus taeda* and *Populus deltoides.*
Advisor: John Cairney
Summary: The pulp and paper industry supports research in genetic engineering with the goal of developing an improved source of raw material. Traditional breeding techniques have been used to successfully achieved this goal in the past. However, the techniques developed by genetic engineering are proving to be more economical than traditional breeding techniques. State and federal agencies strictly regulate the release and use of genetically engineered organisms in the environment. The greatest concern of the agencies is the transfer of selected genes from one organism. It is hoped that sterile organisms will express lower than normal rates of gene transfer.

The goal of the project is to identify regulatory genes expressed in reproductive tissue of loblolly pine and cottonwood trees. These genes will be used to genetically engineer sterile trees for use in the pulp and paper industry. Sterile organisms can be genetically engineered by disrupting the function of regulatory and structural genes responsible for the development of reproductive tissue. Introduction of modified copies of these genes can cause selective tissue death. The desired genes will be identified using information from previously discovered genes with similar known functions. Once the desired genes have been identified, they will be sequenced and isolated.

Michael Sullivan (M. Sc.) * First year student.
Title: Assessing Gene Expression Changes During Culture Cycling
Advisor: John Cairney
Summary: Changes in the settled cell volume of liquid suspension cultures have been observed for many Loblolly Pine genotypes. The volume of settled cells follows a pattern, diminishing to a nadir then rising once more to peak at close to (but often below) the previous high. This cycling may be part of a trend of deterioration; maximum cell volumes never matching a previous high, minimum volumes being lower than the previous low. We wish to learn more about this phenomenon, both to follow the cultures through phases of a cycle and to determine similarities and differences between different peaks, different troughs and between peaks and troughs. Differential Display will be employed as a means of following biochemical changes, as manifest in changes in gene expression. Patterns of gene expression will be determined and potential 'marker' bands will be cloned and analysed.

Byron Waldrop (M. Sc.) * Second year student.
Title: Assessing Somatic Embryo Quality Using Differential Display Techniques
Advisor: John Cairney
Summary: Somatic embryogenesis offers forest geneticists and planters opportunities to multiply superior plants, multiply clones of genetically altered plants, and store genetic information for future plantings. This technique promises to be a powerful tool for insuring an adequate supply of high quality raw material for the paper industry's future. However, the adaptation of somatic embryogenesis techniques to commercially valuable species, such as conifers, has been problematic. It has been difficult to identify high quality embryos, and rates of induction and maturation remain very low compared with those of non-woody plants. Analysis and comparison of gene expression in zygotic and somatic embryos are the keys to understanding these problems. If a gene, or series of genes, are found to be expressed by only a certain quality embryo or only during a certain stage of development, these genes can be used as markers for the classification of other embryos. Comparison of genes that are expressed by zygotic embryos but not by somatic embryo at the analogous developmental stage, may reveal nutritional or environmental imbalances present in the somatic embryogenesis protocol, thus allowing for corrections to be made. The proposed study will use differential display techniques to compare gene expression during developmental stages of zygotic and somatic embryos. Genes whose expression is found to be specific to a growth condition or developmental stage will be investigated further by isolating, cloning, and sequencing cDNA fragments from gels.

The information gathered will then be used to identify high quality embryos, and guide protocol adjustments directed at improving somatic embryo quality.

Vincent Ciavatta (Ph. D.) *
Title: Analysis of Gene Expression During Development of Somatic and Zygotic Embryos.
Advisor: John Cairney
Summary: As a tool to follow Embryogenesis in vitro and in vivo, gene expression during equivalent stages of development will be compared. Differential Display will be used and patterns diagnostic of a particular stage of development for a particular genotype will be generated for somatic embryo. Bands which appear characteristic of early or late development or of a particular stage will be cloned and characterized. Equipped with the technical expertise and physiological insight which this will give us, similar experiments will be conducted with zygotic embryos. Comparing and contrasting gene expression in somatic and zygotic embryos will allow us to evaluate their state of health and permit informed modifications to media which should improve quality and quantity of embryos generated in vitro.

Stephen Van Winkle (Ph. D.) *
Title: An investigation into an unsuccessful tissue culture medium: Determining the role of activated charcoal.
Advisor: Jerry Pullman
Summary: Previous tissue culture experiments with two different activated carbons supplied by Sigma revealed that one carbon promoted embryogenesis of Douglas-fir while the other did not. The goal of this project is to discover why one carbon was ineffective. Research will be directed towards physically and chemically characterizing many (~20+) different carbons with the goal of correlating these characteristics with carbon performance in tissue culture medium. Activated carbon is known to be a versatile sorbent: performance will be measured in terms of sorption of tissue culture medium components (particularly hormone and mineral nutrients). Performance will also be measured using a bioassay model for the Douglas-fir initiation system. This bioassay will be developed using Norway Spruce zygotic and somatic embryos. Initial results indicate that the two carbons have different porosity, different surface charge, and different ionic content. A liquid Norway Spruce initiation system has been successfully demonstrated for use as a bioassay.

Thomas Welt (Ph. D. Requirements completed, will matriculate June, 1997)
Title: Enzymatic de-inking - Effectiveness and mechanisms.
Advisor: Ron Dinus and John Cairney
Summary: Although several theories explaining enzymatic de-inking have been proposed few studies have focused on the mechanism(s) involved. Therefore, the overall objective of the present study is to generate data which will yield a better understanding of the mechanisms involved in enzymatic de-inking. More specifically we will evaluate the effect of enzymatic action on ink and fibers, and how these actions affect ink release during paper disintegration. A well-defined paper material and highly purified enzymes

will be used throughout the study. A technique for visualization of the enzymatic attack on pulp fibers will be developed. Scanning electron microscopy (SEM) will be used to study surface changes caused by enzymatic action. Colloidal gold coated antibodies directed against enzymes or gold-labeled enzymes and transmission electron microscopy (TEM) will be employed to help visualize enzymatic attack on and inside fiber walls. In addition, research efforts will focus on methods to immobilize enzymes. These methods will help to determine: 1) individual effects of a particular enzyme type; 2) if synergistic effects between enzymes are important in de-inking; 3) the spatial distribution of enzymatic attack; and 4) the effect of surface and/or internal action of enzymes on pulp fibers. A literature review based on this dissertation research proposal was published in the February issue of Progress in Paper Recycling.

IPST FOREST BIOLOGY
RESEARCH PROPOSALS
(Awarded, Submitted and in Review or Rejected)

Following is a list of 1995-1997 research proposals which have been submitted and awarded, rejected, or are currently under review. Following the list is a brief abstract or summary from each proposal submitted since our last PAC meeting. If you would like to see any of these proposals please contact one of the IPST authors.

Awarded to IPST

1997 - Currently ranked high for $468,634
1996 - Approximately $ 312,279
1995 - Approximately $ 142,329
1994 - Approximately $ 78,789

Title:	Methods to Assay and Identify Populations of Bacteria Associated with Recycled Containerboard.
Authors (Affiliation):	Gerald Pullman
Awarding Agency:	Containerboard and Kraft Paper Group (CKPG)
Amount Requested:	$42,644
Period of Proposal:	1997
Submitted:	September 17, 1996
Status:	**Awarded, $42,644 to IPST**

Title:	The Role of Peptide Processing in Plant Cell Growth and Development
Authors (Affiliation):	Sheldon W. May (GIT), Gerald Pullman (IPST), and John Cairney (IPST)
Awarding Agency:	Georgia Institute of Technology/Institute of Paper Science and Technology, Pulp and Paper Education Program
Amount Requested:	$ 40,000 ($ 20,000 to IPST)
Period of Proposal:	1996-1997
Submitted:	August 30, 1996
Status:	**Grant Awarded ($20 K to IPST Forest Biology, $20 K to GIT Dept. Chemistry & Biochemistry)**

Title:	The Role of Calcium Dependant Protein Kinases in Xylem Tracheary Element Differentiation and Zygotic Embryos of Loblolly Pine
Authors (Affiliation):	Gary Peter (IPST), and Jung Choi (GIT)
Awarding Agency:	Georgia Institute of Technology/Institute of Paper Science and Technology, Pulp and Paper Education Program
Amount Requested:	$ 40,000 ($ 20,000 to IPST)
Period of Proposal:	1996-1997
Submitted:	August 30, 1996
Status:	**Grant Awarded ($20 K to IPST Forest Biology, $20 K to GIT Dept. of Biology)**

Title:	Molecular Methods for Induction of Early Flowering in Forest Trees.
Authors (Affiliation):	Jeffrey F.D.Dean (UGA), Scott A. Merkle (UGA), Sarah Covert (UGA), Gerald Pullman (IPST) and John Cairney (IPST)
Awarding Agency:	The Georgia Consortium for Technological Competitiveness in Pulp and Paper
Amount Requested:	$230,000 ($120,000 to IPST)
Period of Proposal:	Fiscal Year 1997 (July 1, 1996 - June 30, 1997)
Submitted:	February 1, 1996
Status:	**Awarded $72,000 to IPST**

Title:	Ensuring Fiber Supply Through Biotechnology: New Tissue Culture Tools from Gene Expression Studies.
Authors (Affiliation):	G. Pullman (IPST), J. Cairney (IPST), Jung H. Choi (GIT), Art Ragauskas (IPST)
Awarding Agency:	The Georgia Consortium for Technological Competitiveness in Pulp and Paper
Amount Requested:	$94,075 (IPST), $20,000 (GIT)
Period of Proposal:	Fiscal Year 1997 (July 1, 1996 - June 30, 1997)
Submitted:	13th July 1995
Status:	**Awarded $98,779 to IPST**

Title:	Genetically Engineering Sterility in Commercially Important Southern Trees
Authors (Affiliation):	John Cairney (IPST), Gerald Pullman (IPST), Ronald Dinus (IPST)
Awarding Agency:	The Georgia Consortium for Technological Competitiveness in Pulp and Paper
Amount Requested:	$68,000 (IPST)
Period of Proposal:	Fiscal Year 1997 (July 1, 1996 - June 30, 1997)
Submitted:	13th July 1995
Status:	**Awarded $73,500 to IPST**

Title:	Protecting the Fiber Supply Through Genetic Engineering of Drought Tolerance
Authors (Affiliation):	John Cairney (IPST), Gerald Pullman (IPST)
Awarding Agency:	The Georgia Consortium for Technological Competitiveness in Pulp and Paper
Amount Requested:	$68,000 (IPST)
Period of Proposal:	Fiscal Year 1997 (July 1, 1996 - June 30, 1997)
Submitted:	13th July 1995
Status:	**Awarded $68,000 to IPST**

Title:	Ensuring Fiber Supply Through Biotechnology: New Tissue Culture Tools from Gene Expression Studies.
Authors (Affiliation):	G. Pullman (IPST), J. Cairney (IPST), Jung H. Choi (GIT), Art Ragauskas (IPST)
Awarding Agency:	The Georgia Consortium for Technological Competitiveness in Pulp and Paper
Amount Requested:	$94,075 (IPST), $20,000 (GIT)
Period of Proposal:	Fiscal Year 1996 (July 1, 1995 - June 30, 1996)
Submitted:	13th July 1995
Status:	**Awarded, $75,000 to IPST, Fiscal Year 1996**

Title:	Ensuring Fiber Supply Through Biotechnology: New Tissue Culture Tools from Gene Expression Studies (Matching Funds).
Authors (Affiliation):	G. Pullman (IPST), J. Cairney (IPST)
Awarding Agency:	Georgia Pacific Company
Amount Requested:	$ 10,000 ($10,000 to IPST)
Period of Proposal:	1996 (These Matching Funds are contingent on receipt of grant (same title) from Georgia Consortium in 1995.
Status:	**Awarded January, 1996 ($10 K to IPST Forest Biology)**

Title:	The Role of Peptide Processing in Plant Cell Growth and Development
Authors (Affiliation):	Ronald Dinus (IPST) and Sheldon W. May (GIT)
Awarding Agency:	Georgia Institute of Technology/Institute of Paper Science and Technology, Pulp and Paper Education Program
Amount Requested:	$ 38,990 ($ 19,681 to IPST)
Period of Proposal:	1995-1996
Submitted:	8th August, 1995
Status:	**Grant Awarded ($20 K to IPST Forest Biology, $20 K to GIT Dept. Chemistry & Biochemistry)**

Title:	Methods to Assay and Identify Populations of Bacteria Associated with Recycled Containerboard.
Authors (Affiliation):	Gerald Pullman
Awarding Agency:	Containerboard and Kraft Paper Group (CKPG)
Amount Requested:	$31,329
Period of Proposal:	1996
Submitted:	January 16, 1996
Status:	**To be awarded, $31,329 to IPST**

Title:	Identification and Potential Control of Bacteria Associated with Recycled Containerboard. Upon review, changed to Feasibility study for methods to assay and identify populations of bacteria associated with recycled containerboard.
Authors (Affiliation):	Gerald Pullman (IPST), Ted Heindel (IPST), Alan Rudie (IPST). Proposal changed to one author, Gerald Pullman
Awarding Agency:	Containerboard and Kraft Paper Group (CKPG)
Amount Requested:	$62,000, changed to $6,000
Period of Proposal:	1995-96
Submitted:	March 7, 1995
Status:	**Grant awarded, $6,000 to IPST**

Pending Proposals

Title:	Ensuring Fiber Supply Through Biotechnology: New Tissue Culture Tools from Gene Expression Studies.
Authors (Affiliation):	G. Pullman (IPST), J. Cairney (IPST), Jung H. Choi (GIT), Art Ragauskas (IPST)
Awarding Agency:	The Georgia Consortium for Technological Competitiveness in Pulp and Paper
Amount Requested:	$142,249 ($121,249 to IPST, and $21,000 to GIT)
Period of Proposal:	Fiscal Year 1998 (July 1, 1997 - June 30, 1998)
Submitted:	12th July 1996
Status:	In review, currently ranked high for funding

Title:	Protecting the Fiber Supply Through Genetic Engineering of Drought Tolerance
Authors (Affiliation):	John Cairney (IPST), Gerald Pullman (IPST)
Awarding Agency:	The Georgia Consortium for Technological Competitiveness in Pulp and Paper
Amount Requested:	$84,622 (IPST)
Period of Proposal:	Fiscal Year 1998 (July 1, 1997 - June 30, 1998)
Submitted:	12th July 1996
Status:	In review, currently ranked high for funding

Title:	Molecular Methods for Induction of Early Flowering in Forest Trees
Authors (Affiliation):	Jeffrey F. Dean (UGA), Scott A. Merkle (UGA), Sarah Covert (UGA), Gerald Pullman (IPST), and John Cairney (IPST)
Awarding Agency:	The Georgia Consortium for Technological Competitiveness in Pulp and Paper
Amount Requested:	$355,448 ($180,119 to IPST and $175,329 to UGA)
Period of Proposal:	Fiscal Year 1998 (July 1, 1997 - June 30, 1998)
Submitted:	July 15, 1996
Status:	In review, currently ranked high for funding

Rejected Proposals

Title:	Trees Containing Built-In Pulping Catalysts
Authors (Affiliation):	Gerald Pullman, Don Dimmel, John Cairney (all IPST)
Awarding Agency:	Preproposal - Agenda 2020 - Environmental Group, U.S. Department of Energy.
Amount Requested:	$137,335.65
Period of Proposal:	1997 Fiscal Year
Submitted:	July 7, 1996
Status:	Not Funded

Title:	Trees Containing Built-In Pulping Catalysts
Authors (Affiliation):	Gerald Pullman, Don Dimmel, John Cairney (all IPST)
Awarding Agency:	Preproposal - Agenda 2020 - Capital Effectiveness, U.S. Department of Energy.
Amount Requested:	$137,335.65
Period of Proposal:	1997 Fiscal Year
Submitted:	August 31, 1996
Status:	Not Funded

Title:	Signaling Mechanism that Coordinates Lignin Biosynthesis - A General Approach to Regulated Decreases of Lignin Content in Trees.
Authors (Affiliation):	Gary Peter, Gerald Pullman, John Cairney (all IPST)
Awarding Agency:	Preproposal - Agenda 2020 - Capital Effectiveness, U.S. Department of Energy.
Amount Requested:	$113,407
Period of Proposal:	1997 Fiscal Year
Submitted:	September 9, 1996
Status:	Not Funded

PUBLICATIONS - 1995-1997
(Issued, in press, or submitted)

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